Federation Proceedings

VOLUME 8 *June 1949* NUMBER 2

NOTES ON THE DETROIT CONVENTION OF THE FEDERATION

April 18 to 22, 1949

The thirty-third annual meeting of the Federation of American Societies for Experimental Biology was held at Detroit, Michigan, April 18 to 22, 1949. Headquarters hotels were the Book-Cadillac for three Societies (Biochemistry, Pathology and Immunology) and the Statler for three Societies (Physiology, Pharmacology and Nutrition). All of the scientific sessions of the Societies were held in the Masonic Temple, as was also the Joint Session of the Federation on Tuesday evening, April 19. One motion picture session was presented, at the Detroit-Leland Hotel. The Federation 'Mixer' was held Wednesday evening, April 20, at the Hotel Statler.

Dr. Harry P. Smith, President of the American Society for Experimental Pathology, was Chairman of the meeting and presided at the Joint Session. One hundred and thirteen scientific sessions were held at which 1304 papers were read and 170 papers were read by title. Symposia were presented by the American Society of Biological Chemists, the American Society for Pharmacology and Experimental Therapeutics, the American Institute of Nutrition and the American Association of Immunologists.

The total registered attendance was 3517, including guests (wives of members) and exhibitors. As in previous years, the attendance was about equally divided between Society members and non-members.

Forty-four technical exhibits, occupying 60 booths, were shown.

FEDERATION ACTIONS

The following actions of the Federation Executive Committee are of general interest:

1. The plan to hold the 1950 meeting of the Federation in Atlantic City, New Jersey, April 17 to 21, was confirmed.

2. The Federation assessment for the year beginning July 1, 1949, was set at $3.00 per member of each Society (all members to be included, whether retired, honorary or members of more than one Society). The assessment includes subscription to FEDERATION PROCEEDINGS.

3. It was decided to permit the sale of reprints of abstracts published in the March 1950 issue of FEDERATION PROCEEDINGS provided orders and payment are sent with the abstracts.

4. The Societies approved in principle the proposal for drafting a Constitution, revision of the By-Laws and incorporation of the Federation, the Constitution and By-Laws to be referred to the Societies for approval.

5. The Executive Committee voted that the form of the listing of members in the December issue of FEDERATION PROCEEDINGS be revised and that each entry contain only the following information: name of member; highest earned degree (when applicable both the M.D. and Ph.D. degrees, or, when applicable, the highest earned graduate-school degree and the M.D. degree); mailing address; title of position held (not more than two); and the Society affiliation.

6. The Executive Committee approved the proposal that upon adoption of the new Constitution and By-Laws the Control Committee for FEDERATION PROCEEDINGS be replaced by an Editorial Committee composed of the Society Secretaries and the Managing Editor.

7. The American Institute of Nutrition will act as the host Society for the 1950 meeting and will arrange the program of the Joint Session.

INTERNATIONAL CONGRESSES

Two International Congresses of interest to members of the Federation are scheduled for 1949 and 1950:

a) The First International Congress of Biochemistry will be held August 19 to 25, 1949, at Cambridge, England.

b) The 18th International Physiological Congress will be held August 15 to 18, 1950, at Copenhagen, Denmark.

CORRECTIONS OF ABSTRACTS IN MARCH ISSUE

Page 21. CAMPBELL AND DAVIDSON. The last sentence of the abstract, beginning "The liver slices . . .," should be omitted.

Page 86. KLÜVER. In line 1, the name "Henrich" should read "Heinrich." In line 3, the word "prophyrins" should read "porphyrins."

Page 141. SCHWARTZ, SCHACHTER AND FREINKEL. In line 19, the equation should read "$Z = D - UV - Pw_{In}Pw_{v}$."

Page 144. SELYE AND STONE. Column 1, line 22 from the top of the page, should read, ". . . granules, which is normally produced by such stimuli as folliculoid hormones."

Page 230. MILLER AND BALE. Insert the following table before the last sentence of the abstract.

C^{14} DISINTEGRATIONS/MINUTE/MILLIMOL $\times 10^{-4}$

	TOTAL	RELEASED BY NINHYDRIN	RELEASED FROM NINHYDRIN RESIDUE
Liver			
L-Lysine	4.49		
L-Glutamic acid	0.750	0.145	0.472
		0.147	0.453
L-Aspartic acid	0.21	0.154	0.02
Plasma protein			
L-Lysine	5.28		
L-Glutamic acid	0.607	0.150	0.425
L-Aspartic acid	0.338	0.24	0.02

Page 335. SWINYARD AND TOMAN. Line 16, insert the following table preceding the sentence beginning, "Maximum potency . . ."

R	TD_{50} mg/kg.	ELECTROSHOCK TEST		METRAZOL TEST	
		ED_{50} mg/kg.	P.I.	ED_{50} mg/kg.	P.I.
H (a)	740 (550–1000)	40 (36–44.5)	18.5 (13.6–25.2)	212 (137–324)	3.5 (2.1–5.9)
H (h)	1620 (1095–2400)	170 (111–260)	9.5 (5.3–17.1)	>1600	<1.0
CH_3 (a)	173 (124–242)	17.5 (12.2–25.2)	9.9 (5.2–19.0)	59 (38–92)	2.9 (1.7–5.1)
CH_3 (h)	332 (256–432)	16 (13.0–19.7)	20.8 (14.8–29.2)	203 (151–272)	1.6 (1.1–2.4)
C_2H_5 (a)	170 (129–224)	18 (14.4–22.5)	9.5 (6.6–13.5)	160 (105–243)	1.1 (0.6–1.8)
C_2H_5 (h)	119 (98–144)	10.8 (7.8–15.0)	11.0 (7.5–16.4)	62 (51–76)	1.9 (1.5–2.5)
C_6H_5 (a)	No activity up to 1600 mg/kg.				
C_6H_5 (h)	>3200	30 (20–45)	>100	>3200	<1.0

( ) 95 per cent confidence limits.

Page 335. TABOR. Throughout the abstract the abbreviation mM should read micromol.

Page 344. WAX, ELLIS AND LEHMAN. Line 8, the word "was" should read "were." In line 15, the word "jejunum" should read "intestine." Line 21 (new sentence) to the end of the article should read, "Increased absorption area may have resulted in increased tissue concentration, but did not definitely influence total absorption. The percentage absorption was not markedly affected by the concentration of IPA. Absorption of IPA was 99 per cent complete at the end of 2 hours, the greatest part occurring within the first 30 minutes. Previous absorption of IPA did not significantly influence subsequent absorption from adjacent loops. Ethyl alcohol administered intravenously perhaps decreased the intestinal absorption of IPA."

Page 404. HAUROWITZ AND ETILI. Line 2 from the bottom should read "per antigen-antibody bond or ~ 0.47 kcal. per $Å^2$ results."

Joint Session of the Federation

Detroit, Michigan, April 19, 1949

Chairman: H. P. SMITH

AMINO ACID METABOLISM IN MUTANT STRAINS OF MICROORGANISMS

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THIS discussion of some aspects of amino acid metabolism in microorganism is necessarily limited in scope. It will deal primarily with metabolic relations which have been found to be of general significance in both higher and lower organisms and with certain new relations, probably also of general significance, which have been suggested by studies with microorganisms. In both instances, the discussion will center around the results obtained with the help of mutant strains.

Mutations which have as biochemical consequences defective biosyntheses of essential cellular constituents, such as vitamins and amino acids, are being produced in steadily increasing numbers of types of microorganisms. These now include representative bacteria, yeasts and fungi.

In order to give some idea of how nutritionally-deficient mutant strains of microorganisms are produced and isolated, a brief summary of various techniques now available is given in table 1. Basically, mutations in a microorganism are produced by exposing the culture to the action of a mutagen; x-rays, ultra-violet radiation, or chemicals such as mustard-gas. Pure genetic lines of the treated material are derived from single spores with fungi, or from single vegetative cells with bacteria, by growing them on a 'complete' medium which supplies a variety of growth-factors, both known and unknown. A nutritionally deficient mutant among these cultures is detected by its failure to grow in a 'minimal' synthetic medium; and its defective biosynthesis is identified by testing its growth in variously supplemented synthetic media. This method has been used with *Neurospora* (1) and with *E. coli* (2) and other microorganisms. Several less laborious methods for selecting mutants from large populations have recently been developed. These are also summarized in table 1. All of these modifications are based on the failure of a mutant to metabolize and grow normally without its specific essential supplement. When this supplement is added after a period of incubation, mutant cells of bacteria only then form colonies which at a given time are smaller than those from normal cells (3). Fries has successfully increased the proportion of mutant spores of the mold *Ophiostoma* in mixtures by removing the germinated normal mycelia by filtration (4). With *Ophiostoma* (4) and with *Neurospora* (5) mutants have been visually selected from populations of spores cultured on minimal agar media on the basis that a mutant fails to grow or grows very slowly under these conditions. Even more efficient are methods based on the more rapid death of normal cells due in one case to exhaustion of endogeneous reserves in a minimal medium lacking a growth-factor required by the normal strain (6) and due in the other instance to the growth and consequent sterilization of normal cells in minimal medium containing penicillin (7, 8).

If the B-vitamins and the amino acids are metabolically essential for microorganisms, and if random mutation in nature or in the laboratory results in defective syntheses of these substances, we would expect to find a close correlation between the growth factor requirements of strains as isolated from nature and mutant strains produced experimentally. This expectation is fairly well fulfilled.

The requirements of mutant strains which have now been found in bacteria and fungi do include most of the known water soluble vitamins and the amino acids. This widespread range is summarized most easily by listing those sub-

stances not yet so represented. It can be seen from table 2 that deficiencies in mutant strains of fungi include riboflavin, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid and choline, which are not required by any filamentous fungi yet isolated from nature. So far as is known, folic acid and vitamin B_{12} are not required either by fungi from nature or by mutant strains. For the bacteria, inositol is not yet known to be required but all the other vitamins except riboflavin, folic acid, B_{12} and choline are included in the requirements of mutant strains.

Table 1. Techniques for isolating nutritional mutants of microorganisms

Description of method	Organism used	Reference
Isolation on complete medium, testing on minimal	*Neurospora*	(1)
	E. coli	(2)
Incubated in minimal, later supplemented as desired	*E. coli*	(3)
Incubated in minimal, non-mutants removed by filtration	*Ophiostoma*	(4)
Incubated on minimal, mutants selected by observation	*Ophiostoma*	(4)
	Neurospora	(5)
Incubated in minimal, with selective survival of mutants	*Ophiostoma*	(6)
Incubation in minimal with penicillin, with selective survival of mutants	*E. coli*	(7, 8)

Table 2. Comparison of nutritional requirements of mutant and wild-type strains of microorganisms

Filamentous fungi		Bacteria	
Strains from nature	*Mutant strains*	*Strains from nature*	*Mutant strains*
B-vitamins not required			
B_2	B_{12}	Inositol	B_2
Pantothenic acid	Folic acid	Choline	Inositol
Nicotinic acid			Choline
p - amino benzoic acid			B_{12}
Choline			Folic acid
B_{12}			
Folic acid			
Amino acids not required			
?	Alanine Hydroxy-proline	Alanine Hydroxy-proline	Alanine Hydroxy-proline

A similar comparison for amino acids suggests that in nature few spontaneously arising amino acid-requiring mutants of non-pathogenic fungi survive. However, mutant strains of fungi have been found with requirements for all the well-established amino acids except alanine and hydroxyproline. The same is true for strains of both classes of bacteria.

There is therefore available a wealth of experimental material in mutant strains of bacteria and fungi. In the strains to be considered, for example, single reactions in the biosyntheses of amino acids are blocked by gene mutations in a more specific manner than possible with enzyme inhibitors. The use of mutant strains as tools in the biochemical analysis of synthetic mechanisms is evident. However, before discussing specific examples of this, I want to mention briefly a phenomenon which has been found fairly frequently in mutant strains requiring amino acids. This phenomenon is one of a specific and often extreme susceptibility to growth inhibitions caused by certain amino acids, usually not the ones required for growth. These inhibitions are important both because they may indicate previously unsuspected interrelations between amino acids, and because they must be taken into account in the interpretation of studies of biosyntheses. To illustrate the diversity of antagonistic amino acid relations in Neurospora some examples are shown in table 3. Similar antagonisms have been invoked by Bonner to explain the double requirement of a mutant strain of Neurospora for isoleucine and valine (15). In this case an accumulated precursor of isoleucine appears to inhibit the synthesis of valine, and both are therefore required.

I now want to discuss some of the biochemical interrelations which have been found in amino acid metabolism in microorganisms. Three general considerations should be emphasized for this discussion: first, the validity of the viewpoint of comparative biochemistry, that the biochemical processes of microorganisms, higher plants, and animals are fundamentally similar; secondly, the contributions of studies with mutant strains of microorganisms to the analysis of the biochemical steps involved in reactions and relations between amino acids; thirdly, the im-

portance of mutant strains of microorganisms in discovering hitherto unknown reactions and unsuspected amino acid interrelations.

Since amino acid metabolism has been most thoroughly studied in mutants of *Neurospora* and of *E. coli*, the examples used in this discussion for the most part will come from work with these two microorganisms.

As shown in figure 1, one of the best examples of the essential similarity of amino acid metabolism in different organisms is the synthesis of arginine, in which the same steps of the ornithine cycle are involved in mammals, in *Neurospora* (16), in *Penicillium* (17), and in bacteria (18, 19). Further probable relations of arginine to glutamic acid and to proline are indicated by *reactions 1*, *2*, and *4*. One mutant strain of *E. coli* is apparently blocked at *step 1* and another at *4*, resulting in requirements for glutamic acid or proline, and for proline, respectively (2). A block at *reaction 2* in *Penicillium* apparently explains a requirement for proline or for arginine (17).

TABLE 3. SOME AMINO-ACID ANTAGONISMS IN NEUROSPORA

GROWTH-FACTOR	GROWTH INHIBITOR	REFERENCE
Isoleucine + valine	Excess of either	(9)
Lysine	Arginine	(10)
D-α-aminoadipic acid	Arginine, asparagine, glutamic acid	(11)
Glycine or serine	Asparagine	(12)
Methionine or threonine	Excess methionine	(13)
None (reversed by arginine)	Canavanine	(14)
Histidine	'Complete' medium	(5)
Adenine	Indole	(5)

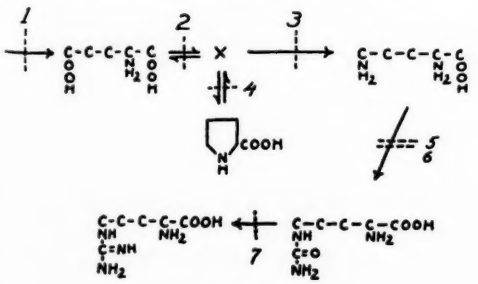

FIG. 1

Another excellent example of the essential similarity in different organisms is in the metabolism of the sulfur-containing amino acids. As shown in figure 2, the relation of cystathionine to cysteine and methionine first established for higher animals by du Vigneaud and collaborators holds also for *Neurospora* (20) and *E. coli* (21). Some minor differences exist, however. In *Neurospora* serine as such seems not to be formed from cystathionine (13). In *E. coli* the reactions leading from methionine to cysteine apparently do not operate in that direction, and this bacterium differs from Neurospora in being able to use D-allocystathionine as a source of methionine (21).

Investigations with mutant strains of *Neurospora* have also indicated unsuspected relations between homoserine and threonine (13), as pictured diagrammatically in figure 3. Strain 51504 grows with either amino acid. A similar relation has been found in mutant strains of *B. subtilis* by Teas (22). An even more surprising relation is suggested by a mutant strain of *Neurospora* with an alternative requirement for isoleucine or threonine (22). These intriguing amino acid interconversions remain to be elucidated.

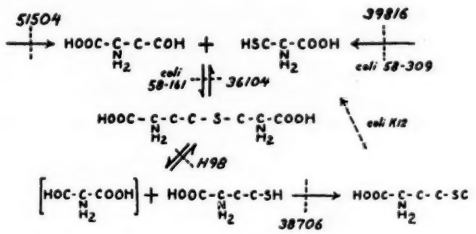

FIG. 2

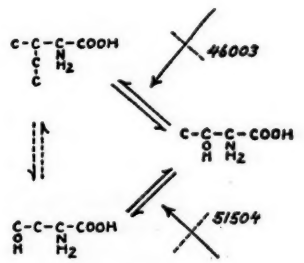

FIG. 3

The metabolic relation between lysine and α-aminoadipic acid found in the rat by Borsook and collaborators (23) and shown in figure 4, has been found also in *Neurospora* since α-amino adipic acid has recently been shown to be a precursor of lysine for strain 33933 (11).

Although antranilic acid, indole, and tryptophane have for some time been known to be met-

abolically related, convincing evidence that anthranilic acid is a precursor of tryptophane was first obtained with mutant strains of *Neurospora* (24). This is shown in figure 5. The mechanism of the conversion of indole to tryptophane has likewise been analyzed with *Neurospora*, and has been shown to involve a hitherto unsuspected condensation of indole with the amino acid serine (25). There seems to be at least two steps involved in the conversion of anthranilic acid to indole, since two genetically different mutants are unable to carry out this reaction. A clue to the mechanism of this conversion has been given by

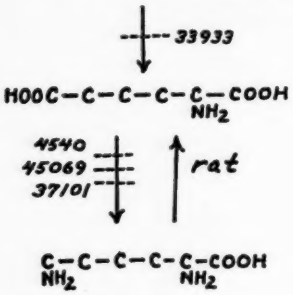

FIG. 4

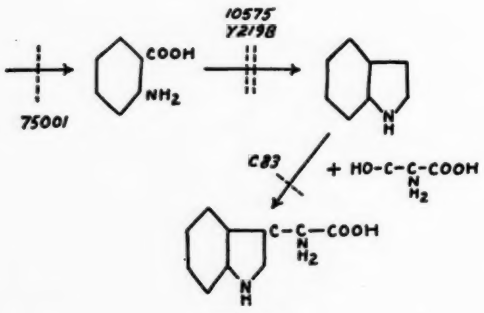

FIG. 5

Nyc *et al.* (26) who have found by isotopic techniques that the carboxyl carbon of anthranilic acid is not present in the formed tryptophane. The conversion of indole to tryptophane has been shown to be an enzymatic reaction involving pyridoxine (27). Recently a mutant strain of *Neurospora* which requires tryptophane and which apparently lacks this enzyme has been described (28). This evidence provides excellent supporting evidence that a gene-enzyme relation is involved in the genetic control of biosynthetic reactions.

An interesting relation between an amino acid and a vitamin is the conversion of tryptophane to nicotinic acid. This relation has been substantiated and clarified with mutant strains of *Neurospora*. As shown in figure 6, some strains of *Neurospora* are known which require either tryptophane or nicotinic acid. This conversion is most readily explained by steps involving kynurenine, the hypothetical compound hydroxykynurenine and the established intermediate 3-hydroxyanthranilic acid. This latter compound is converted to nicotinic acid by strains 40008 and 65001 and by strain Y31881. (29–31). The production of this last intermediate by strain 4540 has been demonstrated by Bonner (31). The evidence obtained with these strains of microorganisms thus lends excellent support to the relation of tryptophane and nicotinic acid suggested first by animal studies. In addition, this conversion has been shown to involve several new

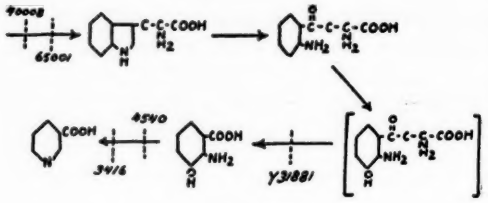

FIG. 6

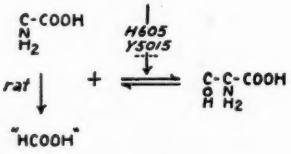

FIG. 7

biochemical reactions of considerable general significance.

Another interesting relation which exists both in animals and in microorganisms is that of the interconversion of glycine and serine. Strains of bacteria (18, 19) and of *Neurospora* (12) are known which have alternative requirements for either glycine or serine. The relation of these two amino acids has been demonstrated in higher animals by Shemin (32) and the mechanism illustrated diagramatically in figure 7 has been recently suggested by Sakami (33). Sakami's evidence, based on the use of marked glycine, suggests that glycine gives rise to formate, which then reacts with another molecule of glycine to form serine. This reaction may be involved in the interconversion of these amino acids in microorganisms. The arrow marked with the two strain numbers on the figure represents the reac-

tion chain leading into this series. There seem to be at least two steps which have been genetically blocked in the synthesis of glycine and serine. Evidence now available suggests that the rat and *Neurospora* may metabolize glycine in somewhat different fashions. Shemin has presented evidence that in the rat glycine is not particularly active in transamination reactions. In *Neurospora*, glycine apparently very readily supplies amino nitrogen for the formation of several other amino acids, including tryosine, histidine, aspartic acid, and glutamic acid, as indicated by the use of nitrogen labelled glycine with a glycine requiring strain (34).

Some other extremely interesting relations between amino acids have been found which deal with the aromatic compounds phenylalanine, tyrosine, anthranilic acid and para-aminobenzoic acid. The precise mechanisms of these interrelations remain to be investigated. As diagramatically represented in figure 8, mutant strains with requirements for each one of the indicated aromatic compounds are known in *E. coli* (18, 19, 35) and in *Neurospora* (24, 36, 37). In *E. coli* recovery experiments have indicated that phenylalanine and tyrosine are synthesized independently, perhaps from a common precursor (35). The synthesis of all four compounds from a common precursor (x) is suggested by the existence of a mutant strain of Neurospora, Y7655, which requires all four substances (36) and by the existence of similar multiple-requiring mutants in *E. coli* (19). The relations existing in these mutant strains provide extremely interesting possibilities for further biochemical investigations of the biosynthesis of aromatic compounds.

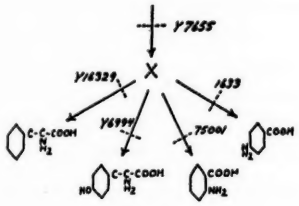

FIG. 8

Shemin (32) has suggested that the non-essential amino acids may be synthesized in the rat by amino acid interconversions rather than by amination of their keto acid analogues. Recent investigations with *Neurospora* have suggested that the keto acid of at least one essential aliphatic amino acid may not be directly involved in its biosynthesis. Working with a mutant strain of *Neurospora* which requires isoleucine and valine (9, 15) E. A. Adelberg, in our laboratory, has recently isolated and identified a new intermediate in the biosynthesis of isoleucine. The structure of this compound and a tentative suggestion of its part in the synthesis of isoleucine are indicated in figure 9. The keto acid analogue of isoleucine is perhaps only indirectly related to the biosynthesis of isoleucine in microorganisms. The evidence for this includes the inactivity of the keto acid in releasing the valine inhibition of growth of wild-type *E. coli*. In addition, some mutant strains of bacteria have been found capable of using the dihydroxy or the keto-acid analogue instead of isoleucine, while other mutant strains can use only the dihydroxy analogue in place of isoleucine. This evidence apparently provides a new clue to the mechanism of synthesis of aliphatic α-amino acids.

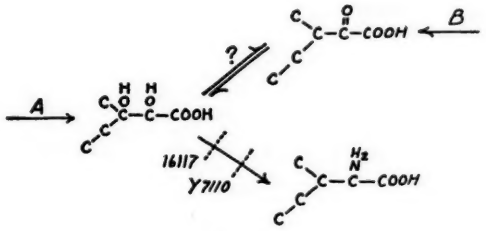

FIG. 9

As mentioned earlier, there is now a considerable body of evidence suggesting that many amino acids may be synthesized by interconversions rather than directly from their keto acid analogues. In addition, evidence is now available that some keto acids are biologically inactive in certain microorganisms. These two lines of evidence cast some doubt on the general significance of direct amination reactions as terminal steps in the bio-syntheses of certain amino acids in microorganisms. Some examples are summarized in table 4. In the first group of examples listed, the most probable syntheses are by way of mechanisms other than amination of the keto acids, perhaps those indicated in the last column of the table. In several instances the keto acids have been found to be inactive for mutant strains of microorganisms. It might be concluded that perhaps the only amino acids which are formed *de novo* by direct amination or transamination are those directly related to known intermediates in

carbohydrate metabolism, notably alanine, aspartic acid and glutamic acid.

Some of the known amino acid relations which have been found to be common to both microorganisms and animals are summarized in table 5. These reactions, which have already been discussed, include those of the ornithine cycle, the relation of cystathionine to methionine and cysteine, the interconversion of glycine and serine, the relation of alpha-aminoadipic acid to lysine, and the conversion of tryptophane to nicotinic acid, and to kynurenine.

TABLE 4. KETO-ACID AND AMINO ACID RELATIONS IN MUTANT STRAINS OF MICROORGANISMS

AMINO ACID	ACTIVITY OF KETO-ACID ANALOGUE	PROBABLE SYNTHESIS FROM:
Arginine	?	Citrulline (16)
Lysine	?	α-aminoadipic acid (11)
Serine	?	Glycine (12)
Glycine	?	Serine (12)
Cysteine	?	Cystathionine (20)
Methionine	+	Cystathionine (20)
Isoleucine	−, +	Dihydroxyacid analogue
Tyrosine	± (10%)	Phenylalanine or common precursor (35)
Aminoadipic acid	− (11)	?
Tryptophane	−	Indole + serine (25)
Histidine	?	
	− (Hydroxy-acid, 34)	?

TABLE 5. REACTIONS COMMON TO MICROORGANISMS AND ANIMALS

Ornithine ⟶ citrulline ⟶ arginine
cystathionine ⇌ homocysteine + [serine]
cystathionine ⇌ homoserine + cysteine
glycine ⇌ serine
α-aminoadipic acid ⇌ lysine
tryptophane ⟶ nicotinic acid
tryptophane ⟶ kynurenine

TABLE 6. REACTIONS AND INTERRELATIONS ESTABLISHED WITH MICROORGANISMS

indole + serine ⟶ tryptophane
threonine ⇌ homoserine
anthranilic acid ($-CO_2+?$) ⟶ indole
kynurenine ⟶ 3-hydroxyanthranilic acid
3-hydroxyanthranilic acid ⟶ nicotinic acid
α,β, dihydroxy, β-ethyl-butyric acid ⟶ isoleucine

As the result of examinations of the mechanisms of these and other biosynthetic reactions with mutant strains of microorganisms, a number of new reactions and relations have been established. These include the reactions summarized in table 6. The involvement of serine in the synthesis of tryptophane has been discussed. The relationship of threonine to homoserine suggested by studies with microorganisms remains to be elucidated. Similarly, the exact steps in the conversion of anthranilic acid to indole are as yet unknown. The essential outline of the biochemical steps involved in the conversion of kynurenine to 3-hydroxyanthranilic acid, and of this compound to nicotinic acid has been suggested. Finally, a role of α,β-dihydroxy-β-ethylbutyric acid in the biosynthesis of isoleucine has been fairly adequately established by studies with microorganisms.

It may be expected that future investigations with microorganisms, particularly with mutant strains, will reveal other new and unsuspected relations between amino acids, and that these strains will prove highly useful tools for the elucidation of the mechanisms of reactions involved in the biosyntheses of these amino acids. It may also be anticipated that further studies correlating enzyme activity with gene mutation will add additional information regarding the role of the gene and of gene mutations in determining enzyme specificity. The first clear-cut example of this relationship in *Neurospora* is the enzyme involved in tryptophane synthesis from indole and serine. Information is now available which should permit the extension of this analysis of gene-enzyme relations to other biosyntheses. These include the syntheses of the amino acids arginine, proline, glycine, and serine. It may be expected that other reactions will become available for such studies as the precise mechanisms of the biosyntheses of other amino acids are established.

In summary, we have discussed various aspects of amino acid metabolism in the examination of which microorganisms have been of primary value Judging from past experience we may anticipate that additional discoveries coming from studies from microorganisms will be of general metabolic significance. The principles of comparative biochemistry thus adequately justify the use of microorganisms in investigations of amino acid

metabolism. In these investigations mutant strains of microorganisms should prove of significant value in future developments.

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VIRAL MULTIPLICATION

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WHEN a virus and a susceptible cell come together, there occurs an increase in the amount of the virus. An increase in the concentration of a virus has not been obtained as yet in cell-free media nor in the absence of living cells. It is now well established that viruses are discrete particles with definite morphology which can be visualized with the electron microscope (1). An increase in viral concentration is associated with an increase in the number of viral particles of like amount; there is a close correlation between the number of particles and the number of infective units (2).

The increase in the amount of virus which occurs in the presence of susceptible cells is generally considered to result from viral multiplication. The validity of this concept was indicated by the early classic work of Woodruff and Goodpasture (3). Apparently, viruses do not carry out demonstrable metabolic activities (4, 5). Moreover, they have not been shown to contain known enzyme proteins (2). If this is true, it seems important to inquire: How do viruses undergo multiplication; where do they acquire the substance and energy necessary for their synthesis; what orients and directs the process? Most workers now think that during their multiplication viruses derive the needed mass and energy from the host cell. But the directional, orienting influence appears to derive from the virus itself even though it may operate through the indispensable partner, the host cell.

Because viruses apparently are multiplied within the host cell and give little evidence of their presence until they burst forth, it is not simple to learn what goes on. Studies with bacteriophages, which are often termed bacterial viruses, have yielded important information. The development by Burnet (6) of a method for measuring the yield of virus from individual bacteria and the development of the one-step growth experiment and other techniques by Ellis and Delbrück (7) made possible close analysis of the multiplication of bacterial viruses. The process is considered as a series of step-wise events. Chance collisions bring the viral particle and bacterial cell together. Attractive forces lead to adsorption of virus at the surface of the cell. One viral particle is all that is required to initiate multiplication, but the cell can adsorb a number of particles equal to the number it can produce (8). Adsorption is rapid and may be essentially completed in a few minutes (9). Adsorption cofactors such as univalent cations (10), tryptophane (11) or calcium may be required. The virus enters the cell, which then rapidly becomes altered with the result that other viruses usually cannot multiply within it (12). At this point mysterious events take over. The virus seems, in effect, to have disappeared as is indicated by the experiments of Cohen and Anderson (13) and Foster (14). This puzzling state, which is termed the latent or silent period, continues for 13 to 40 minutes, depending on the virus employed, when suddenly the virus reappears i.e., bursts from the cell in large numbers (8). As Delbrück (15) has shown, one viral particle leads to the synthesis of 100 to 400 new viral particles in this short interval. Multiplication has occurred, of this there is no doubt, but it happened during the latent period and its stages were unobserved. The results of the numerous elegant experiments which have been carried out provide data about the kinetics of the interaction between bacterial virus and cell; the preliminary events and the final results, but shed no clear light upon the real problem: the biochemical mechanism of viral multiplication.

Results which are closely analogous have been obtained with certain animal viruses, especially the influenza group and mumps. Adsorption by susceptible cells (16–18), the probable nature of certain cell receptors (19), the duration of the latent period and the approximate number of viral particles liberated (20) have been demonstrated. The only striking difference from the bacterial viruses appears in the length of the latent period. With influenza viruses, Henle, Henle and Rosenberg (20) found that this period

is of the order of 6 to 9 hours; with mumps virus it appears to be considerably longer (21).

The riddle of multiplication has puzzled many workers since viruses first were recognized. Various hypotheses have been proposed. With the larger viruses, the psittacosis group as example, the life cycle hypothesis has its proponents, and changes in the size, staining properties and number of intra-cellular viral particles have been correlated with multiplication of the agents (22, 23). Growth and division, as with bacteria, is a possibility but many facts do not fit such a theory. Self-reduplication or autocatalytic processes have been invoked, chiefly since the crystallization of some plant viruses was achieved (24). These processes are hardly less mysterious than viral multiplication itself and do not provide a satisfactory solution to the problem (25). A virus precursor hypothesis has been put forward. According to this view (26) the precursor is present in the cell before infection and is transformed to virus by an autocatalytic process. Another suggested mechanism is the template hypothesis: virus is synthesized by the cell in conformity with the models (the templates) it presents to the intracellular enzymes (25).

The phenomenon of viral interference, perhaps more than any other, has called forth a turn in current ideas. Infection with one virus often precludes infection with another; one virus may act as inhibitor of the multiplication of another. The characteristics of the reaction (27, 28) are consistent with the idea that it is competitive. That it is not dependent upon competition for cell receptors appears established (27, 29), as is the fact that it can be obtained with inactivated virus (30, 31). Taken together, the existing data suggest that the competition may be for a metabolic system in the host cell and that both the integrity and availability of this system are essential for viral multiplication.

If all pairs of viruses showed interference, it could be supposed that all required similar metabolic pathways in the host cell. But numerous pairs of viruses do not show interference; some are capable of simultaneous multiplication in a single cell (32, 33). Because competition does not occur in these instances, it seems probable that non-interfering viruses demand different metabolic pathways of the cell. How many pathways for different viruses there may be is not known but in the cells lining the allantoic sac of the chick embryo it appears there are at least three (21, 29).

Studies on interference have raised the possibility that identifiable metabolic steps may be demonstrable. More detailed information concerning cyto-chemical processes would be of value in unravelling their nature. In the absence of adequate information regarding cellular synthetic processes, attempts have been made to find substances which block viral multiplication and act as specific inhibitors. Knowledge of the chemical nature and structural configuration of such substances may help in identifying the metabolic steps which they block.

Certain acridine compounds have been found to inhibit the multiplication of certain bacterial viruses (34, 14) as well as influenza viruses (35). Foster (14) showed that proflavine concentrations which do not inhibit bacterial growth, inhibit completely the multiplication of T_4 and T_6 viruses. The concentration of inhibitor required is inversely related to the length of the latent period. Of importance is the finding that when introduced during the latent period proflavine effectively inhibits viral multiplication. Inhibition of T_2 and T_6 is obtained when the drug is added 12 to 15 minutes after infection, a period about equal to one-half of the latent period. When proflavine is added later during the period of multiplication, decreased yields of virus are obtained; the extent of inhibition is an inverse function of the time of addition of the drug. Apparently, proflavine blocks some late step in the process of multiplication but does not block earlier steps since removal of the drug during the first part of the latent period permits normal multiplication. Infected bacteria in which viral multiplication is inhibited by proflavine undergo lysis at the end of the normal latent period but do not yield any infective virus (14).

Cohen and Anderson (13) demonstrated that the multiplication of T_2 is inhibited by 5-methyl tryptophane. The inhibition is reversed by the addition of tryptophane (36). As with proflavine, complete inhibition is obtained only if the substance is introduced during the first half of the latent period. These findings raise the possibility that no infective virus is present within the cell during the first half of the period required for multiplication (14). Moreover, they suggest that multiplication is a discontinuous process during which virus particles are built up in steps and that only in the final stage are complete or mature, infective particles developed.

There are three means by which the presence of bacterial viruses can be demonstrated: dependent upon their infectivity, specific antigenicity

and characteristic morphology, respectively. If multiplication occurs in steps, then antigens characteristic of the virus might be present before infective particles are evolved. There is no evidence available on this point with bacterial viruses. However, recent studies by Wyckoff (37) with the electron microscope showed that during the latent period distinct particles are present in the bacterial cytoplasm. At first they are extremely small. They increase in size and number rapidly until they replace much of the cytoplasmic material, but only just before the end of the latent period do they show the sperm-like morphology which typifies the mature, infective virus. These findings support the theory of a step-wise mechanism of viral multiplication.

With influenza virus there is evidence pointing in the same direction. This agent, like a number of other animal viruses, is capable of agglutinating red blood cells and its concentration can be measured by the hemagglutination reaction *in vitro* (38). Gard and von Magnus (39), in studies on the interference phenomenon, obtained startling results which indicate that at least two varieties of viral particles are produced. One variety is incomplete or immature, capable of agglutinating red cells but non-infective and of small particle size with a sedimentation constant of 380S. The other is the classical virus, infective and capable of causing hemagglutination and of usual particle size with a sedimentation constant of 660S. Thus, it appears that influenza virus, during one stage in its multiplication, occurs in a state which is almost entirely non-infective; computations indicate that only about 0.01 per cent of the particles were actually infective (39). These latter particles, however, may be of critical importance and probably are alone capable of initiating the process which results in the development of incomplete, non-infective viral particles. In earlier work Friedewald and Pickels (40) showed that influenza virus preparations contained a component which, although devoid of infectivity, caused hemagglutination and was smaller than the complete virus.

Recently, Hoyle (41) found that antigenic components characteristic of influenza virus, but of small size, are present in infected tissue well before mature, infective viral particles appear. Henle (42) has obtained similar results suggesting that with influenza virus, as with bacterial virus, multiplication may be a step-wise process. The completed viral particle fails to emerge until a series of stages in its synthesis has been completed.

With certain plant viruses equally unorthodox evidence is accumulating. Markham, Matthews and Smith (43) showed that crystalline preparations of turnip yellow mosaic virus contained two components which could be separated by centrifugation. One component is a nucleoprotein and infective, a classical plant virus. The other is non-infective, smaller by 50 per cent than the complete virus and devoid of nucleic acid. Both components react identically with specific antiviral serum, have identical electrophoretic mobilities, crystallize as octahedra and are spheres of similar size. The proteins of both have the same size and shape and contain the same amino-acids in similar proportions. The infective virus contains 28 per cent pentose nucleic acid; the non-infective particle, none. It appears that the presence of combined nucleic acid is essential for the multiplication of the virus. It should be emphasized that no infective virus has been shown to be free of nucleic acid (1, 25).

These observations are indicative of what may happen to viral particles during the process of intracellular multiplication. However, they do not provide information as to the indispensable role of the host cell in the process. We are still confronted by the problems: How does the virus obtain the mass and energy necessary for synthesis; how does it orient and direct cellular metabolic processes?

Cohen and Anderson (44) showed that bacteria infected with a virus stop growing but maintain a constant rate of O_2 consumption. Ultraviolet inactivated T_2 stops the multiplication of *E. coli*. Moreover, ultraviolet inactivated influenza virus inhibits the development of the chick embryo (45). Infected bacteria carry out metabolic processes which give evidence of being abnormal. The most definite abnormalities appear in the synthesis of nucleic acids. Cohen (25) found that *E. coli* infected with T_2 synthesize desoxyribose nucleic acid exclusively, the nucleic acid which is characteristic of the virus. Protein is synthesized from the beginning of infection; DNA synthesis does not begin for 8 to 10 minutes after infection. The available evidence (Cohen 25) suggests that the synthesis of viral peptides precedes and may be essential for the synthesis of viral nucleotides. In Cohen's view (25) the "energy and substance for virus synthesis are

supplied in entirely normal fashion" by the infected cell and the virus "organizes a specific enzymatic environment for its own multiplication." Tests of the hypothesis have not yet been possible with animal or plant viruses.

Studies on the biochemical mechanism of multiplication of animal viruses are beset with serious difficulties. As yet only a very few chemical inhibitors of multiplication have been discovered. Certain complex carbohydrates, polysaccharides, inhibit the multiplication of some animal viruses. A number of polysaccharides derived from bacteria, as well as some obtained from other sources, appear to stop the multiplication of pneumonia virus of mice (PVM) (46). The capsular polysaccharides of Friedländer bacilli are most active of those tested; 2.0 micrograms per mouse is effective. Friedländer polysaccharides also inhibit the multiplication of mumps virus in the allantoic sac; 5 micrograms per egg is sufficient (47). Inhibition of multiplication of either PVM or mumps virus results when the carbohydrate is introduced as long as 4 days after infection.

Friedländer polysaccharides do not cause inactivation of either virus, do not combine with them and do not prevent their adsorption by susceptible cells (18, 46, 47). On the other hand, the polysaccharides are taken up by host cells and remain fixed in them for long periods (21). The extent of inhibition of multiplication is directly related, although not strictly proportional, to the quantity of polysaccharide injected. Moreover, the extent of inhibition is inversely proportional to the amount of multiplication which has occurred, or to the time which has elapsed, before polysaccharide is given. Polysaccharide does not cause any reduction in the concentration of virus present in infected tissue, but further multiplication of the virus is inhibited (46, 47).

Three lines of evidence raise the possibility that inhibition of viral multiplication by Friedländer polysaccharides results from blockade of a metabolic pathway in the host cell: *1*) Inhibition of either PVM or mumps virus multiplication is obtained when polysaccharide is introduced as late as 4 days after infection is initiated. At this time the concentration of virus is so high there can be no reasonable doubt that all susceptible cells are already infected (47). This fact and the finding that inhibitory polysaccharide does not prevent adsorption of virus by susceptible cells appear to exclude the possibility that an extracellular effect is responsible for the inhibition. *2*) The multiplication of mumps virus and PVM is inhibited by Friedländer polysaccharides, whereas the multiplication of influenza and Newcastle disease viruses is not (47). Mumps virus interferes with the multiplication of PVM and, moreover, influenza and Newcastle disease viruses reciprocally interfere with each other, but neither mumps virus nor PVM prevents the multiplication of influenza viruses (29). Thus, viruses which are inhibited by a polysaccharide cause interference with each other but do not cause interference with viruses which are unaffected by the same polysaccharide. *3*) Treatment of Friedländer polysaccharide with alkali results in a product which is not capable of inhibiting viral multiplication. However, such altered polysaccharide blocks the inhibitory effect of the native polysaccharide (21). The blocking action of the altered carbohydrate shows characteristics which are consistent with the idea that it acts as a competitive antagonist relative to the native polysaccharide.

It should be emphasized that only a few viruses have been employed in recent studies on the mechanism of viral multiplication and in each instance the evidence is largely indirect and far from complete. Nonetheless, there are suggestive similarities in the findings with certain bacterial, plant and animal viruses which raise the possibility that they undergo multiplication in like manner. In each instance it appears probable that the viral particle itself may be developed through steps or stages and that the complete or mature, infective particle is chemically more complex than its incomplete predecessors. Of most importance are the indications that only the complete infective particle is capable of initiating the process which results in the development of further viral particles. Speculation as to the identity of the metabolic pathways of host cells apparently required by viruses probably would be unrewarding. But it may be that one step in such a pathway is indicated by the results of studies with chemical inhibitors. As has been pointed out, certain complex carbohydrates inhibit the multiplication of some animal viruses. If these substances are indeed structural relatives, are analogues, of an intracellular substance required in viral multiplication, then it may be that a step in the metabolic pathway which is controlled by polysaccharide is essential. Such an hypothesis has merit only in that it is not contrary to the available data and may provoke further study of the problem.

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THE PHYSIOLOGY OF THE INDIVIDUAL AS AN APPROACH TO A MORE QUANTITATIVE BIOLOGY OF MAN[1]

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THE fact of individual variation is frequently taken to be merely an impediment to biological research. The customary approach is to emphasize those features that are common to all individuals and hope thereby to discern basic relations and general mechanisms. Individual variability is eliminated or minimized by taking enough individuals to get a 'good average.' With laboratory animals the numerical requirement is reduced by using inbred strains or litter mates. With man, where such expedients are almost never available, the task of sampling the whole population would be so great—and actually impossible in view of the unknown distribution of major biological characteristics—that it is really never attempted. We are confined to selected groups which may not represent any large and easily identified segment of mankind and may not even be homogeneous in those respects which are important to the problem. The most popular human guinea pigs, college students, are not necessarily very homogeneous except in regard to chronological age. In any case, the emphasis is on averages without critical definition as to source, on the one hand, and to application, on the other.

This is in sharp contrast with the practice of medicine and the general problem of evaluating individuals as in selecting airplane pilots or professors of physiology. The physician asks how his patient differs from normality; the Air Forces also ask about normality but with a different definition. And, presumably, college presidents have their own inscrutable standards.

It is easy enough to smile at the intuitive and empirical procedures used in medical management and in the selection of candidates for specific jobs. It is easy to be impatient with the non-discriminatory premium rates of insurance companies which presume that all 'non-diseased' persons, except for the obese, have equal disease expectations. As scientists, we should insist that there must be sound bases of a physico-chemical nature which have not been developed and utilized for such judgments. There is a very large area of human affairs in which the day-to-day operations, though concerned with human characteristics, proceed with a minimum of reference to knowledge of physiology, biochemistry, and the kindred sciences represented in the Federation. In our work there seems to have been, so far, relatively little of value for individual prediction.

This may be right and proper; perhaps these applied problems should be of no concern to us. There are three objections to this view. In the first place, if it is agreed that the physico-chemical characteristics of the individual should have predictive value, then those who are most expert at measuring and working with those characteristics should be best qualified to interpret the data. In other words, someone must be concerned with the scientific problem of the individual and in the technical area of physiology the physiologist should take the responsibility.

In the second place, consideration of the individual forces attention on the interrelations between variables, at least some of which must be hidden in the pooled data which refer to the common denominators of all dogs or all men. This means that the scrutiny of the individual should be an instructive point of departure for the study of general relations between variables in physiology and allied fields.

Finally, the experimental biologist, like the clinician, is constantly faced with the problem of deciding what is 'normal.' When does physiology end and pathology begin? But 'normal' function

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may obviously be achieved by a variety of combinations of quantitatively different conditions. Moreover, values for a given characteristic which would be normal for one individual may be abnormal, that is pathological or predictive of future disorder, in another individual, depending on other characteristics and conditions. Once the problem of normality is seriously raised, logic demands successively sharpening and narrowing definitions until one comes to the individual. In essence, the problem is to place, quantitatively, the operating machinery of the individual—function and structure—in the spectrum of possibilities afforded by the species.

The simplest form in which the problem arises is in the evaluation of normality of a measurement. Having measured some characteristic of a person, the question follows: Is the person 'normal' with regard to this characteristic? To illustrate, we may consider the cholesterol in the blood, about which there is considerable interest at the moment. Basal blood samples drawn last December from two members of the laboratory staff, *Mr. X* and *Dr. Y*, were found to contain, respectively, 250 and 310 mg. of total cholesterol per 100 cc. of serum. Are these 'normal'?

Neither cursory nor exhaustive searches of the literature provide the basis for a satisfactory answer, but data recently obtained in the Laboratory of Physiological Hygiene may be analyzed for this purpose. We have values, obtained by duplicate analyses on basal blood samples, from each of 541 'normal' white men, who were leading an ordinary life in a gainful occupation not involving manual labor and who were pronounced free of detectable disease on the basis of medical examination. Their ancestral backgrounds, like that of *Mr. X* and *Dr. Y*, stemmed from the British Isles and Northern Europe. All measurements were made by the same methods and analysts in Minneapolis in the fall and winter months of the year. It will be observed that this material is characterized by an unusual degree of homogeneity as to source—species, sex, race, occupation, freedom from apparent disease, season, geographical location, physiological conditions and analytical method.

Surely it might be concluded that these data should provide a suitable reference standard for *Mr. X* and *Dr. Y*. The mean value for the 541 men is 218.7, with a standard deviation of 50.6 mg. *Mr. X's* value of 250 mg. is only 62 per cent of one standard deviation above the mean and would be expected, on the basis of normal probability, to be exceeded by about 25 per cent of the men in any normal sample like the reference group. *Dr. Y's* value of 310 mg., however, is 161 per cent of one standard deviation above the mean and a value as high as this would be expected to occur in only 5 per cent of the cases. Quantitative placement of *Mr. X* and *Dr. Y* has been made in good statistical terms and the first conclusion is that, with regard to blood cholesterol, *Mr. X* is 'normal' and *Dr. Y* has moderate hypercholesterolemia.

But, having made use of normal probabilities, it is well to check the normality of the distribution of the reference material before closing this chapter. A simple frequency-distribution plot of the data shows a reasonable form but when a true normal curve, calculated from the mean and standard deviation, is superimposed, it appears that the peak is too broad, it is 'platykurtic', and the suspicion is created that the data contained in it are not homogeneous. Obviously, the attempt should be made to segregate the 541 men into more closely defined groups.

TABLE 1. TOTAL SERUM CHOLESTEROL, IN MG/100 ML., IN BASAL REST, IN 'NORMAL' MALES

AGE		NUMBER	CHOLESTEROL	
Mean	Range		Mean	S.D.
19	17–20	109	173.2	±30.2
22.5	21–25	92	179.5	±36.7
32	27–35	17	198.2	±25.3
43	39–44	16	222.4	±25.4
46	45–47	87	242.5	±37.5
49	48–50	98	249.1	±42.8
52.5	51–55	102	251.9	±43.0
68	63–74	20	233.9	±37.4
All ages.....		541	218.7	±50.6

Age was not originally considered because the literature generally disclaims a relation between age and blood cholesterol in adults (1–5). However, Brüger and Mobiüs (6) reported a distinct age trend. In any case, the result of segregating by age groups is given in table 1. Clearly, there is a very large effect of age. Low values in early manhood rise progressively with age, but apparently reach a plateau in the fifties, and slowly decline in the sixties. The implications are numerous and intriguing but are beside the present point which is that, for adult men, it is necessary

to specify age in considering the meaning of a particular cholesterol value.

Mr. X, who is 20 years old, now turns out to have a cholesterol level which is 76.8 mg. above the mean of 109 men in his age group. With a standard deviation of 30.19 mg., such a value would be reached by only 0.5 per cent of the reference population and *Mr. X* must now be labelled as definitely abnormal.

Dr. Y's case is less definite; he is 50 years old and the mean value for 98 men in his age group is 249.1 mg., S.D. = 42.8. A value as high as his (310 mg.) may be expected in only 7.6 per cent of normal men of his age. It is of interest to see whether a more definite result can be obtained by still closer scrutiny of his reference group.

TABLE 2. B.M.R. IN ML. O_2/MIN/M² OF BODY SURFACE, AND TOTAL SERUM CHOLESTEROL, IN MG/100 ML. FOR 'NORMAL' MEN 48–55 YRS. OF AGE

B.M.R. CLASS	NO. OF MEN	B.M.R. MEAN	CHOLESTEROL	
			Mean	S.D.
Higher (118.2–149.3 ml.)	64	126.5	244.5	39.1
Medium (109.4–118.2 ml.)	64	113.6	253.2	40.1
Lower (90.4–109.1 ml.)	63	103.2	256.3	48.4
All (90.4–149.3 ml.)	191	114.5	251.3	42.8

Since we have recorded many characteristics of these men, there is almost an embarrassment of possibilities. As a start, we may consider the basal metabolism, on the ground that blood cholesterol is often high in hypothyroidism. None of these men has myxedema but they do cover a considerable range in basal metabolism—from 90.4 to 149.3 ml. of oxygen/minute/square meter of body surface in the men from 48 to 55 years of age. When the 191 men of this age, for whom full data are available, are arranged in 3 classes according to B.M.R., the result is as summarized in table 2. It is indicated that there is an inverse relation between B.M.R. and the blood cholesterol.

It would be of much interest to continue to sharpen the definition of the 'normal' standard groups, but in spite of the fair size (541) in the original total, the size of the sub-groups is getting small for the present type of analysis. For the next stage we may consider the combined medium and lower B.M.R. classes on the ground that they differ only slightly in their cholesterol averages. These 127 men have been arranged according to relative fatness and divided into two groups with the results shown in table 3.

The criterion of fatness used was the thickness of the skin fold at a specified point on the abdomen, this choice being dictated from the results of rather extensive studies on the estimation of fatness in man. The result would be similar if only relative body weight were used, since this is closely but not perfectly correlated with the skin fold thickness. In any event, in agreement with

TABLE 3. RELATIVE FATNESS, AS ESTIMATED BY THICKNESS OF ABDOMINAL SKIN FOLD, IN MM., ('SKIN'), AND TOTAL SERUM CHOLESTEROL, FOR 'NORMAL' MEN 48–55 YRS. OF AGE WHOSE B.M.R. IS BETWEEN 90.4 AND 118.2 ML. O_2/M²/MIN.

	NO. OF MEN	SKIN MEAN	B.M.R. MEAN	CHOLESTEROL	
				Mean	S.D.
Thinner men ('Skin' 9.5–24.6 mm.)	64	19.2	109.0	249.8	45.7
Fatter men ('Skin' 24.8–60.0 mm.)	63	33.7	108.0	259.7	42.6

TABLE 4. BLOOD CHOLESTEROL AND EYE COLOR IN 127 MEN CONSIDERED IN TABLE 3 (48–55 YRS., B.M.R. MEDIUM TO MODERATELY LOW)

GROUP	N	MEAN	S.D.
Fatter men			
Brown eyes	22	265.8	±37.7
Blue eyes	41	256.4	±45.0
Thinner men			
Brown eyes	27	252.8	±31.9
Blue eyes	37	247.6	±50.4
All men with brown eyes	49	258.6	±38.5
All men with blue eyes	78	252.2	±47.0

advance suspicion, it is indicated that the fatter men tend to have the higher cholesterol values even at the same age and with substantially the same basal metabolic rates.

For a further breakdown it is at least amusing to try one more classification, using an item about which we have no preconceived notions. Eye color is known for all of these men so it may be used to classify further the same 127 men considered in the fatness breakdown summarized in table 3. The results are given in table 4.

In both fatter and thinner men of this age group

the brown-eyed men have slightly higher average cholesterol values than the blue-eyed men. Since both numbers and differences are rather small, we cannot yet conclude that there are real differences in the blood cholesterol related to eye color, but the values given in table 4 are the best estimates so far available for the particular classes of men. Such as they are, they must be used as reference standards in lieu of better ones. We may now return to the evaluation of *Dr. Y* on this basis.

It so happens that *Dr. Y* has a B.M.R. of 106 cc. of oxygen/square meter/minute, and an abdominal skin fold thickness of 32 mm., and his eyes are brown. His blood cholesterol value of 310 mg. is only 44.2 mg. above the mean for his group (first line of table 4) and that mean has a standard deviation of 37.7 mg. According to normal probability, some 12.1 per cent of men in his group may be expected to equal or surpass his blood cholesterol level and he must be judged fully 'normal' in this respect.

The foregoing is an illustration of the result of attempting to evaluate a characteristic of an individual. In the process it was necessary to give more than usual precision to the term 'normal' in the sense of 'average' and free from apparent disease. At the outset no real judgment could be made about *Mr. X* and *Dr. Y* because there were no real standards; much the same could be said about almost any characteristic besides blood cholesterol which might be chosen. In the first phase of the analysis, erroneous judgments were made because sources of variation in the standards were not segregated. The second, third and fourth phases of the analysis revealed a progressively changing picture. Because of exhaustion of the reference standard material, no more discrimination is possible, but it cannot be doubted that the tentative evaluation could be further improved by considering more characteristics in a larger sample. Moreover, it is conceivable that some other characteristics might be more significant and greater discrimination could have been achieved than with the characteristics tested here.

The value of refined standards such as developed here can scarcely be doubted. Equally important is the discovery of relations previously unknown or only suspected. Obviously it is now suggested that more specific research be undertaken with regard to the relations between blood cholesterol and the items considered here, namely basal metabolism, obesity and eye color. The attempt to evaluate the individual provides guidance for other research on mechanisms, though nowhere in this analysis was there any direct consideration of those problems.

Of course, questions of mechanism were in the background when the initial question was asked as to the normality of the blood cholesterol values for *Mr. X* and *Dr. Y*. Currently, such a question is apt to be asked because of the belief that the level of blood cholesterol may influence or participate in the production of atherosclerosis. What *Mr. X* and *Dr. Y* want to know is whether their blood cholesterols indicate anything about their personal ageing processes and inclinations to earlier or later atherosclerosis. The analysis here says nothing about this but we hope that the long range program at the Laboratory of Physiological Hygiene will eventually contribute to this particular prediction problem.

Although the data are not at hand to attempt a prediction analysis regarding cholesterol and atherosclerosis, the general problem may be indicated with another example. On the International High Altitude Expedition to Chile (7) there were 9 men for whom various characteristics were recorded before they went to make a prolonged stay in the high Andes. Though all of the men were 'normal' at sea level, they showed great and consistent differences in their ability to acclimatize to altitudes above 12,000 ft. (3660 meters). Were these strikingly different responses related to the differences in the recorded characteristics of the men at sea level? The analysis of this question may be summarized here because it reveals some of the essentials of the general prediction problem (8).

The differences in the individual characteristics at sea level were reliably established. At high altitude the men lived, ate and worked together, so 'external' circumstances were constant and the large differences in acclimatization must have been predetermined by inherent differences which already existed in the men at sea level. But the items we measured at sea level were not then known to be related to the factors which subsequently produced the different responses at high altitude; there were no clear clues as to which items we should measure. In the end, however, there were two sets of data, one on sea-level characteristics and one on high altitude responses, and the problem was to discover what, if any, were the relations between these.

Each item of measurement at sea level can be separately considered in the first phase of the analysis. Some of the characteristics, including vital capacity, blood pressure, muscle strength

and the Schneider cardiovascular fitness index, appeared to have little or no relation to the ability to acclimatize. But suggestive correlations were obtained with 7 of the variables as indicated in table 5. Ability to acclimatize would seem to be directly related to the alkaline reserve and to the partial pressure of carbon dioxide in the alveolar air at rest, and inversely related to age, to basal pulse rate, to body weight, to the hemoglobin concentration in the blood, and to the partial pressure of oxygen in the alveolar air.

Several of these indicated relations may be surprising but, ex post facto, they are physiologically reasonable. For example, it may be startling at first glance to suggest that a low hemoglobin at sea level should be predictive of high ability to acclimatize to high altitude. But the person who normally maintains a high hemoglobin concentration to meet the sea-level condition of oxygen supply can be considered to have a reduced margin to meet the more difficult situation at high altitude. Similarly, the person who habitually gets along at sea level at a ventilation level which amounts to relative 'underbreathing', may be considered to have a more efficient, or at least more economical, respiratory characteristic. This man will, of course, tend to have, at sea level, a low alveolar pO_2 and a high pCO_2.

TABLE 5. CORRELATIONS BETWEEN OBSERVED ABILITY TO ACCLIMATIZE AT HIGH ALTITUDE AND VARIOUS CHARACTERISTICS RECORDED AT SEA LEVEL BEFORE GOING TO HIGH ALTITUDE. DATA FROM KEYS ET AL. (8)

SEA LEVEL CHARACTERISTIC	CORREL. COEFF.	
Basal pulse rate	−0.61	±0.13
Alveolar pO_2	−0.59	±0.14
Alveolar pCO_2	+0.63	±0.13
Arterial blood alkaline reserve	+0.60	±0.14
Blood oxygen capacity	−0.59	±0.15
Age	−0.53	±0.18
Body weight	−0.42	±0.18

In any case, these correlations indicate that each of these 7 variables has some predictive value for ability to acclimatize. We may inquire as to whether they may be combined so as to provide a more perfect index or measure of the factors which determine the ability to acclimatize. This would seem likely since at least some of the measured variables are not strongly intercorrelated. Four of the characteristics which have the greatest independence may be combined in a least-squares solution for the prediction of acclimatization. The result is:

Acclim. = K + 8.89 Alk. Res. − 2.15 pO_2 − 1.82 age − 1.63 weight (9). The acclimatization ability predicted from this equation is compared, in table 6, with that actually observed. The agreement is good and is statistically significant with the 9 men on whom it was tested.

It could be suggested that, since there were 7 sea-level characteristics which seemed to be related to later acclimatization, a better prediction equation could be evolved by combining all of them in a similar least-squares solution. This may be done, of course, and the final result of computation is given in table 6 (7 factors). But though the 'fit' is slightly better, the computational procedures are more laborious and the statistical significance is actually lessened because of the larger number of coefficients (9).

TABLE 6. OBSERVED RELATIVE ABILITY TO ACCLIMATIZE TO HIGH ALTITUDE AS COMPARED WITH VALUES PREDICTED FROM SEA LEVEL CHARACTERISTICS. DATA FROM KEYS, ET AL. (8) AND FISHER (9)

SUBJECTS	OBSERVED	PREDICTED	
		7 Factors	4 Factors
B	59	61	60
C	32	32	30
D	34	34	32
E	46	45	46
F	65	65	73
H	10	9	11
K	90	89	84
Mc	26	27	28
T	50	51	50

There would seem to be several values in this analysis: *1)* unsuspected relations between variables were revealed; *2)* suggestions were made as to critical items for study in regard to the mechanism of acclimatization; *3)* it was shown that some variables may be profitably combined though otherwise we should not think of them as being associated; *4)* a prediction formula was obtained for trial with other groups. It is reasonable to believe that a like outcome would emerge from a similar systematic attack on other problems of prediction besides the high altitude example cited here.

The two examples used in this discussion, deal-

ing with blood cholesterol and with acclimatization to high altitude, were chosen in part because in both cases there was a minimum of guidance available from considerations of cause and effect and qualitative biological theory. The deliberate choice was made of questions for which frontal attack on the actual questions asked could be attempted, at the present stage of knowledge, only by methods which are largely empirical in a biological sense. Unfortunately, it seems that the development of a true quantitative biology is still so rudimentary that the majority of questions pertaining to the physiology of the individual must be attacked with a similar emphasis on empirical methods; normal cholesterol standards and high altitude acclimatizations are by no means exceptional in this regard. This is not to decry in the least the virtue of a more theoretical approach. Given enough knowledge of theory and quantitative information on the mechanisms, both of the problems discussed here could probably be more easily and surely solved. The point is that such knowledge is now lacking and, as a matter of fact, a good deal of empirical gathering of data, plus rigorous analysis, is necessary to create the wanted body of knowledge.

It should be observed that even when a relation has been mathematically established between two variables, or between a present characteristic and a future development, this does not necessarily mean there is any direct cause and effect relationship. While this is a serious limitation, the fact of some kind of a relationship offers both a challenge and a clue to further study. Moreover, the habit of reasoning solely in terms of likely mechanisms is also objectionable unless supported by direct quantitative evidence. A simple example may be taken from the cholesterol data discussed here.

Lately, the suggestion has been made repeatedly that the development of atherosclerosis may be reduced or delayed by a reduction in the dietary intake of cholesterol on the ground that with a smaller intake there would be less cholesterol in the blood available for deposition in the arteries (10). A good many middle-aged people are being advised by their physicians to avoid eggs, butter, milk and fat meats on this account. Now it so happens that we have data on the habitual cholesterol intake of 312 of the men whose blood cholesterol levels have been examined. These men have been grouped according to their habitual cholesterol intakes and the groupings have been separated according to age. The results are summarized in table 7. Neither in the younger (18–25) years) nor older (45–55 years) men is there any relation between blood cholesterol and the habitual intake of cholesterol, in spite of the fact that the differences in the latter are large.

The purpose of this discussion has not been to present data or even theories, but only to call attention to a point of view and an area of problems. It should be particularly observed in connection with the cholesterol data used to illustrate the argument that it is not, for the present, important whether the relations observed in the men studied at Minneapolis will apply precisely to other groups of apparently similar men. Using the Minneapolis data as a reference—and we must use these, at least temporarily, in default of any other—the best judgment of normality about *Dr. Y* is made from the mean value, and standard deviation, for brown-eyed fat men, aged 48–55, who have medium to low rates of basal metabolism. We have not here asked any questions about the statistical significance of the apparent differences between the various groups segregated according to B.M.R., fatness, age and eye color. These questions are currently being examined, with a larger body of data, at the Laboratory of Physiological Hygiene.

TABLE 7. BLOOD SERUM TOTAL CHOLESTEROL, IN MG/100 ML., AND HABITUAL DIETARY INTAKE OF CHOLESTEROL, IN GM/WK., IN NORMAL MEN 18–25 ('YOUNGER') AND 45–55 ('OLDER')

Younger men					
Number	32	30	30	32	32
Intake limits	1.9	1.9–2.5	2.6–2.9	3.0–3.4	3.4
Intake mean	1.49	2.18	2.64	3.15	4.20
Serum mean	175	180	165	176	184
Older men					
Number	32	30	30	32	32
Intake limits	2.1	2.1–2.5	2.6–3.1	3.7–3.9	3.9
Intake mean	1.62	2.27	2.83	3.56	4.81
Serum mean	242	258	249	253	251

The exploration of the frontiers of useful knowledge in the biological sciences increasingly demands a more quantitative approach which is apparent when specific questions are asked about individuals. Whenever individuals are concerned, it is necessary to consider their variation. Whenever individual variation is examined it is clear

that there are such important interrelations between characteristics that the classical approach of isolating variables into single pairs (dependent and independent) must be used with care. The assumption of homogeneity is too readily made and the custom of dealing only with averages delays the recognition of the total truth.

The goal of all natural science is accurate, quantitative prediction. In the area of human health and disease, this begins with the problem of normality and normal standards. With the extension to development with time, these become more than devices for present classification of individuals and populations; they become the essential apparatus for prediction. But, because of the interdependence of most biological characteristics, simultaneous multi-variable analysis is required. This imposes the burdens of more rigorous selection and characterization of the particular animals and men studied and the use of larger numbers and more complicated mathematical analysis. With all this, the need for true cause-and-effect understanding is not lessened. Pure empiricism has its limitations, no matter how systematic it may be.

We all realize that the development of the biological sciences is still in an early phase and a true quantitative science of biology remains for the future. I have tried to indicate that work on problems of individual physiology must be an essential part of the coming age of quantitative biology. One may approach these problems from the basis of questions about normality or about the prediction of future characteristics; the logical result is substantially the same. Finally, it may be agreed that the fact of individual variability represents far more than a technical obstacle to research; it offers a difficult but intriguing field for research in its own right.

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SOME COMMENTARIES ON ELECTRON MICROSCOPY AS APPLIED IN BIOLOGY

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IT IS now some fifteen years since biological objects were first observed in the electron microscope. In this brief period, during which electron microscopes were made available commercially and a number of important preparative techniques were developed, it has become clear that electron microscopy is destined to play an important role in future biological and medical research. It is the purpose of this paper to discuss some of the technical procedures upon which these advances depend and to mention briefly a few of the contributions which electron microscopy has already made in the biological field. Finally, since this symposium concerns problems of experimental pathology, a few observations will be made concerning the application of electron microscopy in pathology.

RESOLVING POWER AND RESOLUTION

The resolving power of a particular electron microscope is determined by its construction and proper operation. The degree to which this inherent resolving power can be realized in the examination of a particular preparation depends importantly upon the nature of the material in the preparation. For example, if an electron microscope has a limiting resolving power of 25A, resolution of this order may be realized if the particles to be examined are of high electron density (e.g., colloidal gold). However, if the same instrument is used in the examination of relatively large protein fibers the resolution obtainable may not exceed 100–200 A. In this case the large size of the particles and the low electron density of their constituent atoms may make it possible to realize but a fraction of the resolving power of the instrument. If the resolution (distance between centers of the closest particles which can be clearly distinguished as separate) is correctly determined the danger of drawing unwarranted conclusions as to the significance of images seen in electron micrographs will be reduced. There has been some tendency among biologists to infer resolutions considerably greater than the micrographs justify. Indeed, one of the problems of the biologist is to prepare his specimens so as to obtain the maximum possible resolution of the objects of special interest in the specimen.

Some Technical Developments

The procedures of particular interest to the biologist are those which permit him *a*) to obtain the constituent of interest in a form which is most amenable to electron microscopic examination and *b*) to obtain information about thickness and detailed structure of the constituent. Some of the more useful of these may now be discussed.

Thin sectioning. Early attempts (1–3) to obtain sections of cells and tissues sufficiently thin for useful examination with the electron microscope (0.05–0.1 μ) were cumbersome and yielded but a small fraction of suitable sections. The development of high-speed sectioning (4–6) aroused considerable interest among biologists. A number of papers have been published showing electron micrographs of normal and pathological tissues sectioned by this method. However, the results left much to be desired, either because of relatively low resolution or because of poor preservation of the tissue structure.

The theoretical basis for the necessity of high velocity impact of the knife on the specimen has never been convincingly developed. Indeed, it has recently been shown by Pease and Baker (7) that quite satisfactory thin sections may be obtained with the conventional rotary microtome which has been fitted with a simple device to permit very small cutting increments. These authors have obtained electron micrographs of liver cells, muscle and other tissue which are of a quality at least as high as those obtained with the high-speed microtome though in few instances outstandingly superior to those obtained with the light microscope.

As in conventional cytology, the success of thin sectioning in the electron microscopic examination of tissue structure depends very largely

upon the preliminary fixation and embedding. Conventional methods are not suitable and much development will be required since the criteria of success are different than in the case of light microscopy. Doubtless special methods will have to be developed for each type of material. The mere fact that sections may be obtained which are sufficiently thin to be penetrated by the electron beam does not necessarily mean that the electron micrograph obtained will reveal considerably more information at a magnification of 10,000 than was revealed by conventional cytological methods at a magnification of 1,000. This is partly due to the fact that the process of fixation may precipitate materials upon the structures to be examined in a manner such as to prevent resolution of the details of structure within the object.

Thus the problems of fixation may plague the new generation of morphologists who use the electron microscope even more than it did their predecessors who used the light microscope. However, it is to be hoped that the mechanisms of fixation itself may now be amenable to a much more penetrating analysis since the electron microscope is capable of reaching down to the molecular level in visualizing the alterations which proteins and other organic substances undergo when treated with fixatives. At this level the study of fixation is the study of the composition and structure of the protein molecules themselves, a goal worthy of intensive effort.

Fragmentation methods. It was more or less generally assumed that significant advances in the electron microscopy of tissues could be made only by the development of thin sectioning techniques. However, it has already been demonstrated that the use of appropriate fragmentation techniques may yield highly significant results.

In the case of striated muscle it was demonstrated that from formalin fixed tissue fragmented with a blendor, or with sonic oscillations, bits of myofibrils could be prepared which are susceptible of very profitable examination with the electron microscope (8). In such preparations it was possible to resolve the individual actomyosin filaments and to demonstrate that they course in parallel bundles through both the anisotripic (*A*) and isotropic (*I*) bands. This disproved the concept that the relative isotropy of the *I* bands is due to disorientation of the filaments in these regions. It was also shown that, in contraction, the filaments thicken and shorten but are not thrown into coils or folds; hence the contractile elements are considerably thinner than the actomyosin filaments (ca 150 A). There is also evidence for the existence of a periodic variation of density, thickness, or both, of the filaments in the axial direction. The period is of the order of 400 A, which agrees with that obtained by x-ray diffraction studies of muscle (9) and of actin (10, 11). The manner in which this period varies with contraction or tension production has not yet been established with clarity; it is possible that further study of this aspect may throw important light on the contractile mechanism.

Another example of the use of fragmentation methods is provided by recent studies of the nerve axon (12). Frozen sections of formalin fixed nerves were fragmented with sonic oscillations. Fibrous structures were observed in the supernate. The fibrils characteristically have dark edges which suggested the term 'neurotubule.' After staining with phosphotungstic acid or after shadowing with chromium the fibrils manifest an axial periodicity in the form of cross-bands. The period averages about 650 A, which is identical with that of collagen fibrils. This poses a difficult problem. Peripheral nerve fibers are invested with collagen fibers and, since the specimens are prepared by fragmentation of whole nerve it was necessary to demonstrate that the fibrils actually occur in the axon and are not merely extra-axonic collagen fibers which have acquired dark edges by the method of preparation, perhaps by adsorption of dispersed myelin material. The view that the fibrils are axonic in origin was supported by the fact that they were observed in fragmented axons isolated from the giant fibers of the squid and by the fact that after nerve degeneration the fibrils could no longer be observed (13).

This example illustrates the difficulty of localizing structures obtained after fragmentation of whole tissues. The difficulty happens to be particularly emphasized in the case of nerve because of the close similarity of the presumably intracellular structures with those of the extracellular collagen fibrils. The sampling difficulty is very prominent in this problem.

Tissue cultures. Another method of observing intracellular structures without the use of sectioning is that of tissue cultures. The cells are grown in a thin film so that they tend to flatten out and remain quite thin. When the culture has reached the desired stage the cells are fixed and transferred to metal grids for observation in the electron microscope. Various types of struc-

tures have been observed, including the limiting cell membrane, mitochondria, particulates of different kinds and, in certain malignant cells, dense particles which may be viruses (14–16). Fixation is also critical in such work not only as to the kind of fixative but also the duration of fixation; the structure may be strikingly different after 15 minutes' fixation in osmic acid from that after fixation for 45 minutes.

With the current development of tissue culture techniques, particularly with respect to synthetic media, it is probable that the number of cell types and problems which may be studied by this method with the electron microscope will be greatly increased.

Staining. One of the most useful techniques employed by the histologist is that of elective staining by means of which he is able not only to achieve heightened contrast between tissue constituents but also to obtain information about the chemical nature and electric charge of the structures which combine with the stain.

Stains are also valuable in electron microscopy. However, in this case the heightened contrast depends on the electron density of the stains rather than their color; they are essentially 'electron stains.' They are useful to the extent that they can attach molecules containing atoms of large weight to specific tissue constituents or to localized regions in these constituents. Biological materials are composed predominantly of atoms of low weight. Therefore some stains customarily used in histology may be useful as electron stains because of the presence of a few atoms of higher weight. These stains have not yet been extensively explored. Rather, emphasis has been placed on substances which contain atoms of great weight (17). Particularly useful are the coordination compounds of the heteropolyacids. Osmic acid, which was early used in electron microscopy, is also an effective stain. Phosphotungstic acid (PTA) has been extensively used in this laboratory though phosphomolybdic and uranyl phosphotungstic acids are also effective.

Electron stains have been used very successfully in bringing out the fine structure of fibrous proteins. Particularly striking is the case of the fibrils of the adductor muscles of molluscs. Unstained fibrils show usually only an irregular, mottled appearance. After staining with PTA, a periodic cross-banding, with spots occurring in a regular array along the bands, was observed (18). This structure agrees remarkably closely with that deduced from small-angle x-ray studies (19). Detailed intraperiod banded structure was also observed in collagen fibrils after staining with PTA.

Electron stains may function both in a specific and a non-specific manner. Thus PTA may combine with specific basic groups in proteins depending on *p*H, concentration and so on. To what extent such specific affinities may reveal the location of particular chemical groups in the structure stained remains to be seen. As usually used, electron stains probably act in a relatively non-specific manner, essentially revealing local differences of thickness. Detailed intraperiod structure with six or more bands can be observed in collagen fibrils stained with PTA (20–22). However, under favorable conditions, similar structure though with low contrast, can be seen in unstained fibrils. From metal-showed specimens it was concluded that the bands which stain with PTA are regions of relative thickness. It is possible therefore that the heightened contrast obtained with PTA is due in part at least to a non-specific volume effect.

It seems quite possible that certain of the cytochemical procedures developed for the localization of specific chemical groupings, enzymes and so on, may be applicable in modified form in electron microscopy. The writer has been investigating this possibility in the case of the acid phosphatase reaction which involves the formation of any insoluble lead salt about the structure containing the enzyme. In this case the heavy metal would constitute an electron stain for this specific enzyme. Success in such investigations will probably depend on the degree to which the reaction can be made truly specific.

Heavy metal shadowing. This technique, developed by Williams and Wyckoff (23, 24), has proven enormously valuable in biological as well as in most other types of electron microscopy. When properly applied, shadowing reveals extremely small details of structure. Indeed, already in the early days of its application in the study of viruses, thin protein filaments and even globular protein molecules themselves, the inherent structure of the film on which the specimen is deposited became a limiting factor. The search for a film which is essentially structureless to within the resolving power of the electron microscope is still in progress. In the meantime, film difficulties may be partially avoided by depositing the material in sufficient thickness to cover the

film completely and observing the discontinuities on the surface of the material. Precaution must be taken to avoid aggregation of the metal atoms by the action of the electron beam during examination in the electron microscope. Thus gold has a strong tendency to aggregation and this has given rise to erroneous conclusions in some instances where this metal was employed. Such difficulties are minimized by the use of metals like chromium, platinum and uranium.

Replicas. Plastic replicas were originally developed for the study of the surface structure of metals and other opaque objects. Excellent electron micrographs have been obtained of the structure of tooth and bone (25–27). With the early successes with replicas it was hoped that the method might be applied to sections of tissues. However, thus far little has been accomplished in this direction; the chief difficulty is in successfully removing the plastic film from the tissue.

In its application to the study of the finest details of structure the replica technique suffers somewhat because of the finite size of the plastic particles and because of alterations which occur either to the specimen or to the replica at one or another of the steps in the procedure. Resolutions greater than 50–100 A are seldom achieved. By shadowing the replica film very slight differences in elevation are brought out.

While it is impossible to observe biological structures in their normal aqueous environment in the electron microscope, it is possible to obtain replicas of moist material and in this way to get some information of the surface structure. This has been done in the case of bacteria in their culture medium (28–30) and also with collagen fibrils (31). Replicas of dried collagen fibrils show the characteristic periodic surface elevations corresponding to the cross-bands. Replicas of slightly moist fibrils show a smooth contour. This is consistent with the x-ray diffraction results (32) which indicate that, unlike dry collagen, the moist collagen fibril is essentially a smooth cylinder; loss of water from localized regions probably gives rise to the periodic axial contour of the dried fibrils. In applying the moist replica technique it is of course important to control accurately the water content of the specimen.

The replica technique which, like that of metal shadowing, has no counterpart in standard techniques of histology, will probably prove more and more useful in biological problems as technical difficulties are overcome.

SOME CONCEPTUAL CONTRIBUTIONS

To what extent has the infant field of electron microscopy made significant contributions to our concepts of the molecular organization of cells and their constituents? It is obvious that observations of importance to the physiologist or pathologist may be made at resolutions only two or three times greater than that obtainable by the light microscope (500–1,000 A). However, it is in the colloidal range of about 25–100 A that the most significant advances may be expected in the next decade.

The direct visualization of animal, plant and bacterial viruses has been of great academic and practical value. Thus far relatively little progress has been made in the study of the detailed internal organization of viruses though x-ray studies (33, 34) offer some clues in the case of the plant viruses. The details of the process by which viruses undergo self-duplication have not yet been penetrated though a beginning has been made in this important problem by Williams and Backus (unpublished).

The investigation of chromosome structure has fared less well probably because chromosomes are difficult to prepare in a condition best adapted to electron microscope study at high resolution. Recently, from a study of thin sections of the chromosomes of *Drosophila*, Pease and Baker (35) identified certain particles with the genes. However, until more definitive reasons for this identification are adduced and higher resolution obtained, such claims must be considered with caution.

Electron microscopy has contributed substantially to our knowledge of the structure of the fibrous proteins. Since fibrous arrays play a dominant role in many vital cellular processes, the investigation of their structure, their origin in cells and tissues and their alterations in physiological processes stand high in the priority list of problems in this field.

One of the most intriguing of the properties of protein fibers is their large repeating periods which may extend to hundreds of Angstrom units both in the axial and transverse directions. X-ray and electron microscope evidence for such periods has been obtained for collagen, keratin, myosin, actin, fibrin, protozoan trichocysts and several other proteins.

These regular repeating periods serve as 'fingerprints' by which particular fibrous proteins may be identified in tissues. However, the period in

itself may suffice to characterize only the *class* of protein. To identify the particular member of the class, it may be necessary to study the detailed intraperiod structure. Thus a number of proteins, differing widely from each other in chemical composition, belong to the 'collagen class' (36). From small-angle x-ray studies (37) it has been shown that, while members of this class all have approximately the same large axial period, the intraperiod structure varies from one to the other member of the class. This discovery may prove of great interest in attempts to devise a 'molecular taxonomy,' and perhaps 'phylogeny,' of the proteins.

Aside from their identifying value the repeating patterns may be expected to reveal something about the internal organization of the fibrous proteins. Since the bands and sub-bands repeat with very great regularity, there must be an underlying regularity of molecular organization.

From present evidence it appears that collagen is composed of a parallel packing of extremely thin filaments; possibly the polypeptide chains themselves represent the unit fibrous structures. The filaments have the inherent pattern of axial periodicity; to the extent that the neighboring filaments in a fibril are in register laterally with respect to the periodic structure, the whole fibril will appear to be cross-straited.

The keratin-myosin-fibrin class of fibrous proteins appears to have a somewhat different structure. Thus fibrous actin is made of a linear aggregation of globular actin molecules (10, 11, 38, 39). The reversible transformation from the fibrous to the globular modification is not only of great physical chemical interest in itself but may be involved in muscle contraction (40).

The structure of fibrin is of unusual interest in connection with the theory of the causes underlying the production of long repeating periods. Physical chemical measurements indicate that fibrinogen molecules have dimensions of about 35 × 700 A. Yet fibrin fibrils have a very accurately repeating pattern of about 250 A (42) and fine structure has been observed within the bands (42). In aggregating to form fibrin, how do fibrinogen molecules give rise to a repeating period bearing no obvious relation to molecular length or composition? It seems clear that such phenomena depend upon interactions of submolecular units. Hall (42) has suggested that such interactions must involve colloidal forces, e.g. forces exerted over considerable distances. This type of spatial adjustment of small particles so as to produce a macro-pattern of great regularity is fundamental in the production of structure in protoplasm. Alterations in these adjustments, due to changes in chemical environment, are probably at the basis of many physiological phenomena. The underlying physical forces which determine regular inter-particle spacings, as in tactoids, have been under investigation and some clarification of the various possibilities has been achieved (43).

ELECTRON MICROSCOPY IN PATHOLOGY

The rapidity with which electron microscopy will be profitably applied in the study of the structure of abnormal tissues and of the phenomena underlying pathological alterations will of course depend upon the development of new techniques which will have to be specially devised for the purpose. However, assuming that adequate techniques were at hand, it should be stressed that before abnormalities can profitably be studied, the investigator must be thoroughly familiar with the structure of the normal tissue. Such familiarity will probably require years of work with each special tissue. Particularly vexing is the sampling problem when working at such high magnifications. Unless one is able to show that adequate sampling of the specimen has been achieved, and some rough quantitative relations established, it is difficult to demonstrate with conviction that a particular difference of structure exists between the normal and abnormal specimens. 'Normal' specimens usually show a fairly wide range of variability, depending on conditions of preparation. The extent of these variations must be at least roughly known.

Reference was made above to the fairly complex technical aspects of electron microscopy. It should not be supposed that useful application of electron microscopy to biological and medical problems can be achieved only by physicists fully conversant with electron optical theory. On the other hand it should be clear that the biologist entering the field should be prepared to spend the time required to become thoroughly familiar not only with the operation of the instrument and with the accessory techniques, but also to learn the criteria by which his results may be correctly evaluated and interpreted.

Though the difficulties to be surmounted by the biologist in applying this technique to his problems may appear to be great, the rewards that await the deeper penetration of the structural organization of protoplasm are correspond-

ingly great. The expected advances will be accelerated to the extent that the lessons of the past century of histology, with its uninterpretable artifacts, are needed and, wherever possible, the full knowledge of modern biophysics and biochemistry is brought to bear on the problems.

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American Institute of Nutrition

Symposium on Nutrition in Preventive Medicine

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NUTRITION AND DENTAL CARIES[1]

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BOSTON, MASSACHUSETTS

THE extensive breakdown of the teeth and their supporting structures among modern populations represents a real challenge to investigators in the broad field of public health. Some idea of the extensive destruction and loss of teeth produced by these two major causes of dental disability can be obtained from the annual cost of dental services in the United States. In 1945, approximately $652,000,000 was spent for dental care, almost all of which was for restorative work (1); this figure actually represents 48.7 per cent of the cost for physicians' services and 13.1 per cent of the total outlay for all medical services. The Veterans Administration alone, in the last three months of 1948, spent a total of $11,519,000 for restorative dentistry in World War II veterans (2). Since estimates have been made that the 83,000 practicing dentists in this country can care for little more than 25 per cent of the existing dental needs, the above figures represent only a part of the true picture (3, 4). Urgently as these figures indicate the need for preventive knowledge, their message seems all too weak when one is confronted in the clinic with a child in early adolescence with such rampant caries that complete extraction and full dentures are indicated.

Although technical procedures in dentistry developed through several centuries have attained a high degree of restorative perfection, not one of these gives the least promise of effecting a reduction in the incidence or rate of progress of dental disease. Undoubtedly, the greatest hope for the prevention or retardation of either tooth decay or diseases of the supporting structures lies in the collection of fundamental knowledge about the various factors which influence the development and maintenance of the dental tissues. A wide variety of investigations need to be conducted to study the effect of various systemic conditions on *1*) the differentiation, development and maturation of the teeth and their supporting structures (the gingival margin, the epithelial attachment of the gingiva to the enamel, the cementum, the periodontal membrane and the alveolar bone), *2*) the metabolic processes in the mature, hard dental structures, in the supporting structures, and in the pulp and, *3*) the rate of secretion, quantity and composition of saliva. A wide variety of the experimental tools and methods in the field of nutrition are readily applicable to study the relation of nutritional influences to the above dental problems. In order to evaluate the results of these investigations, full cognizance must be taken, wherever possible, not only of the length of the experiment and character of the diet during the experiment but, also of the state of development and condition of the teeth and their supporting structure, the previous history of these tissues, the ability of the experimental subject to utilize the food provided, and the hereditary constitution and the physical well-being of the control and experimental individuals, regardless of whether they are human subjects or laboratory animals.

Time does not permit an extensive historical

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résumé of the early literature on the relation of nutrition to dental caries, but it is of interest to comment on a few of the important investigations conducted in the past with human subjects. Early research in nutrition clearly related the nutritional value of the diet consumed during the developmental period to tooth structure. These findings prompted much speculation and research on the relation of nutrition, in particular of vitamin D, both during tooth development and after their emergence into the oral cavity on the development of tooth decay in children. The experiments conducted by Mellanby in the early 30's (5) indicated that the administration of vitamin D to institutionalized preadolescent children resulted in the development of significantly lower numbers of new carious lesions than were observed in the control children. These results have since been confirmed by other investigators (6, 7). The only negative results were obtained in experiments where older children were used (6, 8, 9).

Greater reductions in the dental caries attack rate were observed by Boyd and co-workers in diabetic and in normal children fed adequate diets over long periods (10). These reductions were attributed by the investigators to the nutritional adequacy of the diet. Certain experiments pointed strongly to the maintenance of a favorable calcium and phosphorus retention (11, 12). Careful dietary counselling of outpatients has been reported by Howe, White and Elliott (13), and by Becks and Jensen (14) to give reductions in the occurrence of new lesions by a magnitude of 60 to 70 per cent. Several investigations with children have been conducted in which the results were interpreted as indicating that increases of the soluble carbohydrate content of the diet resulted in increases in the initiation and rate of development of new carious lesions in fully formed and in recently erupted teeth (15, 16). The latter investigators have felt that the reductions in dental caries experience observed by Boyd and co-workers should be attributed to the low carbohydrate content of the experimental diet and not to their nutritional adequacy. From these conflicting backgrounds arose the current controversy as to the primary cause and methods of controlling tooth decay to which there is presently no complete answer.

Much more knowledge about the effects of nutrition during tooth development and maturation upon the susceptibility to dental caries is needed in order to explain numerous exceptions to the current hypotheses concerning the susceptibility to tooth decay. A valuable demonstration of a developmental relationship between diet and caries resistance is provided in a large volume of dental statistics which have been methodically collected in the school clinics of Europe. Sognnaes (17) has assembled and analyzed available reports on the trends in dental caries experience among European children during the past forty years. A total of 27 surveys were evaluated which included observations on three-fourths of a million children from eleven European countries; Czechoslovakia, Denmark, England, Finland, France, Germany, Holland, Norway, Scotland, Spain and Sweden. Most surveys contained data which had been collected by trained examiners at yearly intervals over a period of several years and which had been tabulated by narrow age groups for limited geographical districts. Thus, the dental caries experience of the children of a given age in any year in these communities could be compared to that of the children of the same age in any other year. These excellent aggregations of material were studied for possible effects of dietary changes which were enforced on entire populations during World Wars and in the recovery periods thereafter. Because of the long duration of some surveys, the relation of dietary changes to the susceptibility to tooth decay could be evaluated in much greater detail than would be possible in the best controlled human experiments of shorter duration. In the summary of the trends in dental caries experience recorded in these surveys, Sognnaes concluded that the teeth of European children showed a definite and relatively uniform tendency to a decreased incidence of tooth decay toward the latter part of and following both World War I and II. The reductions in tooth decay were most significant in young children and in those teeth of older children which developed and/or matured during the war years. After the initiation of wartime dietary regimens, there seemed to be several years' delay before an appreciable reduction in the susceptibility of individuals to tooth decay was observed. Following the first World War there was an even greater delay in the return to the prewar susceptibility to tooth decay. Insufficient time has elapsed since the recent war to determine how long the present low dental caries attack rate will continue.

As an illustration of the time relationship between dietary changes and susceptibility to tooth decay, the dietary and dental data collected by several groups of Norwegian investigators during World War II permits the best analysis of the difference in reduction of tooth decay noted between those teeth which developed during war years and those which had erupted before, or shortly after, the beginning of hostilities. The annual sugar consumption of Norway was listed for 1936 as 36.3 kilograms (kg.) per person. In 1939 before Norway's entrance into the war, sugar was rationed in Norway to 17.9 kg. per person per year. This 50 per cent reduction apparently did not significantly reduce the susceptibility of the permanent teeth of 7-year-old children to caries. Through the years 1941 to 1945, inclusive, the average consumption per year was relatively constant at about 13.5 kg., a reduction of about 60 per cent below the prewar level. In addition to the sugar, large amounts of highly refined white flour were consumed by the Norwegian peoples in the prewar period. An estimate was made that 1242 calories of the average daily caloric consumption by an adult were supplied by refined white flour, and an additional 357 calories from sugar, i.e. more than 50 per cent of the daily calories were provided by refined carbohydrates. With the beginning of the occupation, there was a complete substitution of high extraction flour for the refined white flour and, simultaneously, drastic reductions in the amount of meat, dairy products, eggs, fruits and some vegetables. The loss of calories from these sources was largely made up by cheaper vegetables, mostly kohlrabi and potatoes, wartime bread, and fish when and where available.

The dental statistics for the Norwegian survey are summarized in table 1. For the purposes of the present comparison, the best age for observations of the initiation of carious lesions in the deciduous teeth was at 2½ to 3 years of age at which time the deciduous teeth have recently erupted into the oral cavity; a similarly suitable age for observation of the caries incidence in permanent teeth was in the 7-year-old group where the only permanent teeth were ones which had recently erupted. Unfortunately, data on the incidence of caries in the deciduous teeth of the 2½- to 3-year-old group were not collected for the years 1940 to 1943, inclusive. However, by 1944 there had been a 65 per cent reduction in the number of carious teeth and a 44 per cent reduction in the number of children with dental caries. In 1945 and 1946 there were, respectively, 80 and 88 per cent reductions in the number of carious deciduous teeth, and 66 and 80 per cent reductions in the number of children with dental caries of the deciduous teeth. A more detailed evaluation is available for the permanent teeth of the 7-year-old children (figure 1). Negligible reductions in the number of carious teeth were observed during the first 2 years of the war.

TABLE 1.[1] TIME LAG BETWEEN RATIONING OF REFINED CARBOHYDRATES AND REDUCTION IN DENTAL CARIES OF NEWLY ERUPTED TEETH (EXPOSED TO A SIMILAR ORAL ENVIRONMENT FOR A COMPARABLE LENGTH OF TIME)[2]

Norway

YEARS OF WAR		PERCENTAGE REDUCTION IN CARIES FROM 1939 LEVEL				PERCENTAGE REDUCTION FROM THE PREWAR CONSUMPTION OF:			
		Reduction in Carious Teeth		Reduction in Children with Dental Caries					
		Deciduous teeth (2½–3 yr.)	Permanent teeth (7 yr.)	Deciduous (2½–3 yr.)	Permanent (7 yr.)	Flour[3]		Sugar[4]	
		%	%	%	%	%	kg.	%	kg.
	1939	—	—	—	—	—	(120)	50	(18)
1	1940	(?)	6	(?)	0	35	(78)	70	(10)
2	1941	(?)	7	(?)	0	39	(73)	62	(13)
3	1942	(?)	23	(?)	0	39	(73)	62	(13)
4	1943	(?)	42	(?)	0	39	(73)	62	(13)
5	1944	65	57	44	7	39	(73)	62	(13)
6	1945	80	75	66	8	24	(91)	62	(13)
	1946	88	(?)	80	(?)	(? on the increase)			

[1] From SOGNNAES, R. F. *Am. J. Dis. Child.* 75: 792, 1948.
[2] Figures for the caries are averages from the youngest age groups recorded in Norwegian surveys 18, 20, 22, 23 and 24 shown in original table 1.
[3] The use of refined flour was restricted before the invasion, and limited to babies and sick people after the occupation (April 1940). The extraction of the flour was increased from 69% before the war to 85% already in 1939, and soon after 95% of the grain was utilized for flour production.
[4] The percentage reduction of sugar is calculated from the 1936 conumption of 36.3 kg. per individual per year. The severest rationing, 10 kg. per individual (i.e., 70% below prewar), came into effect late in 1940. From then on until 1945 Toverud (by adding periodic increases) has estimated a weekly consumption of 260 gm. (i.e., 13.5 kg. per year or an average of 62% reduction from the prewar level).

Then major consecutive reductions were observed in 1942, 1943, 1944 and 1945, amounting to reductions of 23, 42, 57 and 75 per cent below the 1939 figure. In other words, the reduction in carious permanent teeth in 1945 after 6 years of war was higher than in any other previous war year. No reduction in the percentage of children with dental caries of the permanent teeth was noted until 1944 and 1945 when there were

7 and 8 per cent less children with tooth decay. A comparison for deciduous teeth can be made from the Norwegian data of Toverud collected in the towns of Blaker and Fet. The reductions in dental caries in the deciduous teeth of 2-year-old, 4-year-old and 6-year-old children are tabulated for the years 1939, 1944, 1945, 1946 and 1947 in table 2. Data were not available for 1940 to 1943, inclusive. The children in these three age groups examined in 1944 had the least reduc-

amined in 1945 and 1946 had 64 per cent less carious deciduous teeth than 4-year-old children in 1939. In 1947 there was a slightly lower reduction (55%). In the 6-year-old groups of children where the deciduous teeth have been exposed to the oral cavity for 4 to 5 years, the least reduction (21%) in dental caries experience was observed in 1944. In 1945, 1946, 1947 the reduction in carious deciduous teeth had increased to 44, 47, and 56 per cent, respectively. The

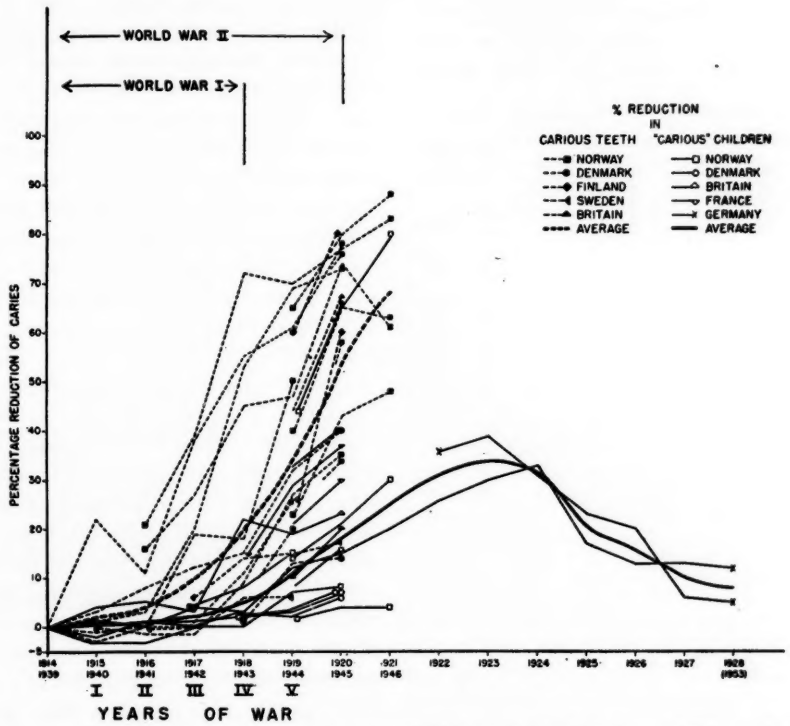

FIG. 1. YEAR-TO-YEAR REDUCTION of caries-susceptible teeth and carious children during the two World Wars. (From SOGNNAES, R. F., *Am. J. Dis. Child.* 75: 792, 1948.)

tions in dental caries experience even though their deciduous teeth had been exposed to the oral environment induced by wartime diets for 1, 3 and 5 years, respectively. In the 2-year-olds maximum reduction (87%) in caries-affected teeth was observed in the children examined in 1946, i.e. born in 1944 where the teeth developed in 1944 and 1945. By 1947, there was a slight increase in the dental caries attack rate. Even in 1947, the 2-year-old children had 74 per cent less carious deciduous teeth than in 1939, this despite a gradual return toward prewar diets in the two years following the cessation of hostilities. In the 4-year-old groups, the children ex-

deciduous teeth of the 6-year-old children examined in 1944 had been developed in prewar years and appeared to have profited little by wartime dietary changes. However, those teeth in 6-year-old children examined in 1947 were formed in the years 1941–42 and had profited greatly even though the last 2 years of their oral exposure had been influenced by post-war diets.

The question arises as to the adequacy of the diet of the Norwegian children throughout the war years. Relatively good data have been collected by Stoltenberg (18) who reported that the caloric requirements of the children from birth to the age of 7 years were adequately provided.

From 7 to 20 years of age there was an increasing caloric deficit from an average of 370 for the youngest to 1260 in the oldest. All public school children were stated to be taller in 1943 than in 1935. Underweight children were observed in prewar years at a rate of 16 per cent of the child population; this figure had only increased to 24 per cent in 1943. Therefore, it appears unlikely that the reductions in tooth decay were due to any restriction in the caloric consumption. In addition, Ström (19) studied the food habits of 102 families in Oslo and two smaller localities who were able to maintain a daily average of 2,847 calories (2708 to 3046) per person per day throughout 1942 to 1945, inclusive.

TABLE 2.[1] DEVELOPMENT AND POSTERUPTIVE CONDITIONS OF DECIDUOUS TEETH OF NORWEGIAN CHILDREN DURING AND AFTER WORLD WAR I[2]

AGE	YEAR EXAMINED	YEAR OF BIRTH	YEARS OF DENTAL DEVELOPMENT	YEARS OF ORAL EXPOSURE	CHILDREN WITH CARIES	REDUCTION IN CARIES-AFFECTED	
						Children	*Teeth*
					%	%	%
2	1939	1937	1937–1938	1938–1939	75	—	—
2	1944	1942	1942–1943	1943–1944[3]	42	44	65
2	1945	1943	1943–1944	1944–1945	25	67	78
2	1946	1944	1944–1945	1945–1946	15	80	87
2	1947	1945	1945–1946	1946–1947	34	54	74
4	1939	1935	1935–1936	1936–1939	100	—	—
4	1944	1940	1940–1941	1941–1944[3]	86	14	38
4	1945	1941	1941–1942	1942–1945	79	21	64
4	1946	1942	1942–1943	1943–1946	70	30	64
4	1947	1943	1943–1944	1944–1947	74	26	55
6	1939	1933	1933–1934	1934–1939	100	—	—
6	1944	1938	1938–1939	1939–1944[3]	97	3	21
6	1945	1939	1939–1940	1940–1945	95	5	44
6	1946	1940	1940–1941	1941–1946	95	5	47
6	1947	1941	1941–1942	1942–1947	87	13	56

[1] From SOGNNAES, R. F. *Am. J. Dis. Child.* 75: 792, 1948.
[2] Interpretation of chart by Toverud from examinations at Blaker and Fet.
[3] The groups whose teeth after eruption have been exposed to an oral environment favored by the greatest wartime reduction in refined carbohydrates. Yet these are the groups which show the least reduction in caries. A 'developmental' explanation is suggested in text.

The marked reductions in dental caries experience among European children during the latter years of World Wars I and II cannot be explained entirely on the basis of an oral environmental effect produced by a reduction in the refined carbohydrate content. The sudden onset of restrictions in the refined carbohydrates available for distribution and the constant amounts available for rationing in Norway throughout the years 1940 to 1944, inclusive, eliminates any argument that the reduction in dental caries incidence was gradual and prolonged because of a gradually increasing restriction in refined carbohydrates. If the oral environment, as influenced by the composition of the diet, had been the preeminently important factor in determining the dental caries experience of these children, the reduction in tooth decay should have been evident within a year or two in all children with recently erupted teeth regardless of whether the teeth were formed before, at or after the initiation of the wartime dietary regimen. In view of the long delay in the production of a reduction in dental caries experience Sognnaes (17) believed that the wartime diets appeared to have permitted the formation and maturation of teeth which were less susceptible to the development of carious lesions. Even when these teeth were exposed to the oral environment produced by post-war diets, they were highly resistant to decay. If the delay in caries reduction had only been observed in Norway in World War II, reasonable doubt might be cast upon this interpretation; since similar trends of rather uniform magnitude had been observed in ten other European countries during World War I and II, the relationship of wartime diets during tooth development to an increased resistance to dental caries appears to be well established. Despite the conclusiveness of the above relationship, there has been an almost universal tendency on the part of the European investigators who collected these dental statistics to interpret them as strong evidence of oral environmental changes without preliminary influences on the tooth structures.

There is a real need to continue to collect survey data of this type in the hope of determining how long the reduction in caries continues and whether any future change can be correlated with dietary alterations. Parallel to the collection of more human statistics, many investigations with laboratory animals are needed to evaluate the exact role of diet during tooth development. During the period from 1910 to 1945, inclusive, animal experimentation in the dental field did not keep pace with human investigation in quantity and much less in quality. The white rat was used frequently in experiments designed to test human findings with unpredictably confusing

and conflicting results. In the middle of this era, Hoppert, Webber and Canniff (20) observed that various lesions developed readily in the molar teeth of the white rat only if the experimental diet were composed largely of coarse particles. When the coarse particles were ground finely, dental caries did not develop. Thereafter investigators demonstrated that the coarse particles caused a mechanical injury to the enamel surfaces followed by tooth decay (21). When the molars of the upper jaw were extracted,

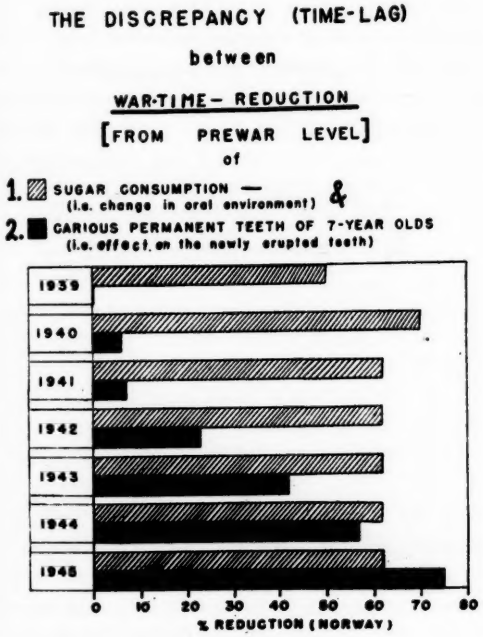

FIG. 2

prior to administration of the coarse particle diet, practically no lesions developed in the lower molars (22, 23). Since human caries is not believed to result from such mechanical causes, this type of regimen was untenable.

In the last seven years, satisfactorily controlled experimental circumstances have been devised for the study of tooth decay in four species of experimental animals: the Syrian hamster (*Cricetus auratus*), the cotton rat (*Sigmondon hispidus hispidus*), the common laboratory rat (*Mus norvegicus*) and the rhesus monkey (*Macaca mulatta*). In 1942, Arnold (24) observed that carious lesions developed in the molar teeth of hamsters which were fed finely ground, high carbohydrate diets composed largely of natural foodstuffs. Two years later, the molar teeth of cotton rats which had been maintained for periods of 10 to 14 weeks on purified rations were found to have been susceptible to the initiation and development of carious lesions (25). The composition of a typical caries-producing, purified ration is given in table 3. The incidence of tooth decay was particularly high in the third molars which were largely developed during the experimental period and which did not erupt until late in the experiment. Tests on the effect of masticating this ration made by the extraction of opposing molars indicated that there was a much less important effect of mastication upon the initiation and progress of carious lesions in this species than had been observed previously in the white rat fed the caries-producing, Hop-

TABLE 3. A TYPICAL CARIES-PRODUCING, PURIFIED RATION

	gm.		*mgm.*
Sucrose	67	Thiamine	0.35
Casein	24	Riboflavin	0.35
Corn oil	5	Pyridoxine	0.35
Salts	4	Calcium pantothenate	2.0
1:20 liver concentrate powder	2	Niacin	2.5
		Choline chloride	100.0
Whole liver substance	2	Inositol	100.0
	—	p-aminobenzoic acid	30.0
	104	β-carotene	1.1
		α-tocopherol	5.0
		2-methyl-1,4-naphthoquinone	0.6
			I.U.
		Irradiated ergosterol	310

pert-Webber-Canniff diet (22, 23). Further evidence that the carious lesions in cotton rat molars were not preceded by mechanical injury was obtained through the examination of ground sections of teeth and jaws after varying periods of experimental exposure. In addition, if all ingredients in the purified ration were finely powdered, the rates of initiation and development of carious lesions were not altered (26).

Examples of the need for careful control of all possible variants in caries investigations with laboratory animals are presented in tables 4 and 5. In the former, an effect of caloric restriction to produce a reduction in the initiation and development of carious lesions is recorded (27). In the latter, the results of two experiments to study if there were any effect of the type of cage and bedding upon the dental caries experience are tabulated (26). Those cotton rats housed

in screen-bottom cages had a higher dental caries attack rate than littermates maintained in cages where the sheet metal floors were covered with shavings. On the contrary, cotton rats housed in cages where the sheet metal floors were covered with paper clippings did not have any different caries experience than littermates kept in cages with screen bottoms. The causes of the effects on dental caries experience occasioned by caloric restriction and by the type of bedding are unknown, but the effects are sufficiently great as to necessitate careful control.

TABLE 4. EFFECT OF CALORIC RESTRICTION

AMT. OF RATION 100	NO. OF RATS	WT. GAIN	NO. OF CARIOUS LESIONS		EXTENT OF LESIONS	
			Mean	C. R.[2]	Mean	C. R.[2]
		gm.				
A) Controls fed *ad libitum*	9	73	26.1±2.2[1]	3.1 (a,b)	79±9	4.0 (a,b)
B) 65% of amt. controls received	11	32	17.5±1.8	5.2 (a,c)	35±6	6.6 (a,c)
C) 50% of amt. controls received	6	16	12.0±1.5	2.4 (b,c)	20±3	2.1 (b,c)

[1] Standard error of mean. [2] Critical ratio.

TABLE 5. AVERAGE NUMBER AND EXTENT OF CARIOUS LESIONS OBSERVED IN COTTON RATS MAINTAINED FOR 14 WEEKS IN CAGES WITH DIFFERENT TYPES OF FLOORS AND BEDDING

TYPE OF BEDDING	NO. OF COTTON RATS	AV. NO. OF CARIOUS LESIONS	S.E.M.[1]	C.R.[2]	AV. EXTENT OF CARIOUS LESIONS	S.E.M.[2]	C.R.[2]
None....	7	33.7	1.3	6.0	110+	6+	6.1
Wood shavings.....	10	17.8	2.3		39+	8+	
None.....	9	27.2	2.8	0.9	80+	12+	0.9
Paper clippings...	10	24.1	2.)		67+	8+	

[1] Standard error of mean. [2] Critical ratio.

Sognnaes (28) has studied the caries-conducive effect of the above purified diet when fed to rodents during, as well as after, tooth development. If weanling rats, hamsters and mice born to females fed laboratory chow throughout the reproductive cycle were fed the chow diet for periods of as long as 9 months after weaning, practically no carious lesions developed in the molar teeth. When weanling white rats and hamsters born to mothers maintained on chow throughout pregnancy and lactation were fed the purified ration for 4 to 8 months, a few carious lesions developed in their molar teeth. In contrast, when female rats and hamsters were changed from chow to the purified ration at parturition and their offspring maintained on the latter diet for 3 to 5 months after weaning, the molar teeth of the offspring developed a rather high number of carious lesions. A still higher rate of dental caries experience was observed in offspring of female rats and hamsters which had been fed the purified ration throughout pregnancy and lactation. The increases in number of molars and number of areas affected by the carious process, and the increase in caries score which were observed for the Syrian hamster are presented in figure 3. In the mouse similar but less striking developmental effects of the purified ration during tooth development were observed.

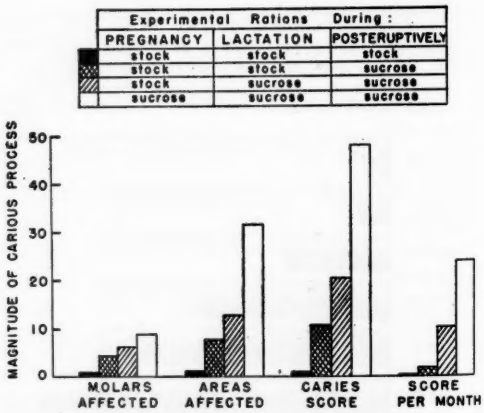

FIG. 3. CARIOGENIC EFFECT of a purified experimental diet (labelled sucrose ration) during tooth development (Syrian hamster). (From SOGNNAES, R. F. *J. Am. Dent. A.*, 37: 676, 1948.

There is a very strong parallel between these studies with the rodents and the above interpretation of the trends in dental caries experience of European children. The white rat and the hamster appear to be suitable experimental animals for the evaluation of dietary influences during tooth development. One possible drawback to the use of these species is the fact that the crowns of the first and second molars are in an advanced state of development at birth and the third molars are largely formed at weaning. Thus, any

dietary effect on these stages of development has to be mediated through the maternal organism via the placenta and/or the milk. This does not seem to be a serious difficulty in view of the qualitative effect of diet during tooth development already described.

One advantage of the cotton rat in developmental studies is the early weaning age (14 days) at which time the third molar crowns are in an early stage of development. The remainder of their formation can be influenced by diets begun at weaning. Another illustration of the inadequacy of the purified diet for the development of the dental structures has been observed through this method of studying the developmental rate of the third molar in the cotton rat. Offspring maintained for 100 days after weaning on the purified diet had a retarded rate of eruption and root formation in the third molars. Among 854 cotton rats killed at this age, the upper third molars of 77 had not pierced the mucosa; an additional 81 had emerged into the oral cavity but were only partially erupted. In contrast, the upper third molars of all cotton rats maintained on natural diets until the 54th postnatal day, or longer, were fully erupted. A similar but less severe retardation in the development of the lower third molars of cotton rats fed the purified diet was observed (26).

Various studies have been made of methods for altering the initiation and rate of development of carious lesions in fully developed molar teeth of weanling cotton rats and common laboratory rats which had been bred for high caries-susceptibility. When representatives of the susceptible strains of both species were maintained for appropriate periods after weaning on the basal purified ration, a high caries attack rate was observed. If diets were fed in which the level of fat, or protein, or both were increased at the isocaloric expense of sucrose, appreciable reductions in dental caries experience were observed (26, 29). If the sole source of nutrients were mineralized whole milk, no carious lesions developed in either species of highly susceptible rodents; as much as 10 per cent of sucrose or other soluble carbohydrates by weight could be dissolved in the mineralized whole milk without any appreciable increase in the dental caries experience (26, 29). At least part of the reduced development of carious lesions observed when milk diets were fed has been shown to be due to its fluid nature (30). Thus, there are numerous dietary procedures in rodents by which development of dental caries after tooth formation is largely completed can be influenced.

As an extension of these studies to primates, species of monkeys have been sought which were susceptible to dental caries. Although the rhesus monkey maintained on natural diets throughout life has an extremely low incidence of tooth decay, representatives of this species maintained on purified diets for long periods have been found to develop a moderate number of lesions (31). The following experiment has been conducted to determine whether certain factors which influence the development of tooth decay in rodents would have the same effect in primates. Twelve rhesus monkeys, purchased from an animal dealer after capture in their native environment, have been maintained from the time they were about 1½ years of age for an additional 3½ years on highly purified rations (32). The 6 monkeys maintained on ration I, which contains 73 per cent sucrose, have not developed any extensive degree of caries. In 2, no caries has been found by oral examination and radiographs. Four have small, slowly progressing carious lesions in the first permanent molars which were almost completely formed at the beginning of the experiment. The second permanent molars and the first and second bicuspids have been formed to a large degree while the monkeys were maintained on the purified ration and have recently erupted. Insufficient time has elapsed since their eruption to permit development of detectable carious lesions. The 3 monkeys maintained on ration II, in which all sucrose has been replaced by fine dextrin, have only developed a few minute lesions. One is caries-free, and 2 have slowly progressing lesions in the first permanent molars. The three monkeys maintained on ration III, in which one-half of the sucrose has been replaced isocalorically by lard, have as yet developed no carious lesions.

In contrast to the above monkeys, 4 rhesus monkeys were transferred to purified ration IV containing 64 per cent sucrose at a much younger age and have in a much shorter period developed definite carious lesions even in the generally less susceptible deciduous dentition. Two were born in captivity in the colony of the Obstetrics Department of the Yale Medical School. Both developed definite carious lesions in the deciduous teeth within a few months after the purified ration was first fed. The other two, one male and one female, while born in the wild were transferred to the purified ration at the time of weaning. In

both cases, the second deciduous molars have definite carious lesions after a year's maintenance on the purified ration, during which time their weight has doubled.

The demonstration of the above contrast seems to merit considerable further investigation. The fully formed teeth of the older monkeys, reared in the wild, appear to have a great caries-resistance, which even the high sucrose ration with the demonstrated ability to produce a high dental caries attack rate in caries-susceptible rodents, has been unable to fully overcome in 3½ years. True, even the older monkeys maintained on this ration exhibited more tooth decay than comparable monkeys maintained on natural diets. However, none of the older monkeys attained the degree of caries observed in the 4 younger monkeys which were transferred to the purified ration regimen at an earlier period of development.

In summary, there appears to be little question about the importance of nutrition during tooth development and maturation in determining the susceptibility to dental caries. At the present stage of the investigations, there is insufficient data to justify speculation as to the identity of the active factor, or factors, which mediate this effect or the mechanism of operation. In most future studies, it will be necessary to define the stage of development of the teeth under investigation and wherever possible, the dietary circumstances which preceded the experimental period. Both human surveys and animal experimentation justify the conclusions: *1*) teeth can be developed by proper nutritional regimentation which are highly resistant to tooth decay under extreme caries-producing circumstances, and *2*) teeth which are highly caries-susceptible at the time of emergence into the oral cavity can be protected from the carious process to some degree by dietary methods. It is scarcely necessary to emphasize that our present knowledge in this subject can be considered as little more than a fundamental introduction into a complex and controversial problem. The prevalence of dental caries and its sequelae, inflammation of the pulp, cysts, abscesses and further focal and systemic infections indicate the necessity for extensive exploration of the relationship of nutrition during dental development, maturation and maintenance.

The author expresses his appreciation for the contributions of his colleague, Dr. Reidar F. Sognnaes, to the investigations described above and to the preparation of this review. We are indebted to the John and Mary R. Markle Foundation, the Nutrition Foundation, Inc., the Sugar Research Foundation, Inc., and the United States Public Health Service for financial support of various projects.

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AMINO ACID REQUIREMENTS OF MAN[1]

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THE isolation and identification of threonine (1, 2) rendered it feasible, for the first time, to support growth upon diets carrying appropriate mixtures of highly purified amino acids in place of proteins. By the successive removal from such diets of single amino acids, the nutritive role of each was established (3). The results demonstrated that, for the growing rat, ten amino acids are essential dietary components. These are valine, leucine, isoleucine, threonine, methionine, phenylalanine, tryptophan, lysine, histidine, and arginine. The exclusion from the food of any one of these, other than arginine, leads to a profound nutritive failure, loss in weight, diminished appetite, and eventual death. In contrast to these spectacular effects, arginine deprivation merely decreases the rate of gain (4). This is accounted for by the fact that arginine can be synthesized by the rat (5), but not at a rate which keeps pace with the needs of the organism for maximum growth. This behavior distinguishes arginine from the amino acids of the non-essential group. The latter apparently can be manufactured in sufficient amounts to meet fully the requirements of the animal.

The above experiments provide much information concerning the types of synthetic reactions which the animal organism can and cannot accomplish. However, the ultimate objective throughout our investigations has been the establishment of the amino acid requirements of man. It was anticipated that time and expense might be saved by first conducting the animal tests, and then applying the results thus obtained to the more difficult and costly studies upon human subjects. Subsequent events have fully justified this supposition.

Investigations in the human species were first undertaken in this laboratory during the autumn of 1942, and since then have been in progress continually. Publication of the detailed findings has been postponed deliberately in order to permit the accumulation of a large number of data upon a comparatively large number of individuals. It was hoped thereby to diminish the probability of errors in interpretation. The results now appear to be sufficiently numerous and definite to warrant positive conclusions.

Healthy male graduate students have served as the experimental subjects. In formulating the diets, only those articles of food were selected which are virtually devoid of nitrogen of an unknown nature. These consisted of corn starch, sucrose, butterfat (which had been melted and centrifugated to remove particles of protein), corn oil, inorganic salts, and vitamins. The starch, corn oil, inorganic salts, and parts of the sugar and butterfat were mixed with water and baked into wafers. The remaining portions of butter and sugar were consumed separately, the former as a spread for the wafers, and the latter as a flavoring agent for the amino acids. The vitamins were supplied in the form of daily doses of cod liver oil, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide, ascorbic acid, calcium *d*-pantothenate, α-tocopherol, 2-methyl-1,4-naphthoquinone, and a concentrate of liver (6) equivalent to 5 gm. of Wilson's 'liver powder 1:20.' The amino acid mixtures furnished 6.7 to 10 gm. of nitrogen daily, and were taken in solution flavored to taste with filtered lemon juice and sugar. From such a diet, the daily intake of nitrogen of an unknown nature amounts to 0.30 to 0.35 gm.

The results of the *qualitative* tests have already been summarized elsewhere (7), and need not be repeated here. The details of the procedures and findings will be published at a later date. In the meantime, it is sufficient to present the final classification of the amino acids. This is shown in table 1.

As will be observed, the amino acids which are required by adult man for the maintenance of

[1] The investigation herein described was made possible by liberal grants from the Nutrition Foundation, Inc., and the Graduate College Research Fund of the University of Illinois.

nitrogen equilibrium are identical with those previously found necessary for the maximum growth of the rat, except in the case of histidine and arginine. The findings with respect to arginine are not surprising inasmuch as the adult rat (8) and the adult dog (9) have been shown to be capable of manufacturing this amino acid at rates commensurate with its use for maintenance. On the other hand, the observation regarding histidine was quite unexpected. This amino acid is required by all higher animals thus far tested. Despite this fact, its exclusion from the diets of the human subjects induced no change whatsoever in the state of the nitrogen balance. At first, the results were viewed with skepticism. It was suspected that histidine had found its way into the food despite the intensive purification of the amino acids. Careful tests excluded this explanation. Furthermore, repetition of the feeding trials with other subjects led to identical conclusions. To date, 42 individuals have been kept in nitrogen balance upon diets which were devoid of this amino acid. Evidently, histidine, like arginine, is not an essential dietary component for the maintenance of nitrogen equilibrium in human adults. Whether the growing child, like the growing animal, requires histidine, arginine, or both of these amino acids, must await the results of further investigations. Moreover, one cannot yet exclude the possibility that certain amino acids which are not necessary for nitrogen equilibrium in normal subjects under ordinary circumstances may become indispensable during disease, or for special body functions such as detoxication, reproduction, or lactation.

TABLE 1. CLASSIFICATION OF AMINO ACIDS WITH RESPECT TO THEIR ROLE IN MAINTENANCE OF NITROGEN EQUILIBRIUM IN NORMAL ADULT MAN

ESSENTIAL	NON-ESSENTIAL
Valine	Glycine
Leucine	Alanine
Isoleucine	Serine
Threonine	Cystine
Methionine	Tyrosine
Lysine	Aspartic acid
Phenylalanine	Glutamic acid
Tryptophan	Proline
	Hydroxyproline
	Histidine
	Arginine
	Citrulline

Having established *qualitatively* the amino acid requirements of normal man, *quantitative* studies were next undertaken. The objective in the latter was to discover the minimum intake of each of the 8 essentials which is capable of permitting nitrogen balance when the diet furnished sufficient quantities of the other 7, and an adequate supply of nitrogen for the synthesis of the non-essentials. In the following pages attention is directed particularly to this aspect of the problem.

Before proceeding further, the reader should be appraised of certain limitations inherent in nitrogen balance studies, particularly when applied to the establishment of minimum requirements. The first of these is associated with the daily fluctuations in nitrogen output encountered in investigations of this sort. Regardless of how accurately one may control the nitrogen intake, and how diligently he may prescribe the routine physical activities of the subjects, moderate day to day variations in the excretion of nitrogen are observed almost invariably. This fact is not limited to experiments involving amino acids. It is equally true of subjects upon protein diets. Perhaps the explanation is to be found in slight alterations in the daily rate of metabolism or of excretion, or possibly in the degree of muscular tonus. In any event, the factors responsible appear to be beyond the present range of experimental control. Certainly, the fluctuations to which we refer are not to be accounted for by errors in the collection of the excreta. In order to minimize alterations in nitrogen loss via the perspiration, all of our tests were terminated before the onset of hot weather.

These variations due to unknown factors are of little consequence when one is making a qualitative experiment. The effects exerted by the exclusion of an essential amino acid from the food are so profound that they are unmistakable. However, after a moderate change in the intake of an indispensable dietary component, the alteration in the nitrogen balance is usually correspondingly small. Therefore, in all of our quantitative experiments the policy has been adopted of inducing a distinctly negative balance at some stage of the test, and then raising the intake of the amino acid under investigation until a consistently positive balance, as measured by the average for a period of several days, was attained. In this fashion, daily fluctuations are leveled, and the results are exceedingly clear-cut. In most

instances, no attempt has been made to establish the minimum level more accurately than within 0.1 gm. of the L amino acid.

Another observation which has proved to be somewhat disconcerting is the difference in the minimum amount of an amino acid which is required by different individuals under comparable experimental conditions. Originally, we had hoped that two quantitative experiments with each amino acid would suffice to establish the minimum requirements of man. This assumption was soon found to be incorrect. With certain amino acids, the results are quite consistent from one subject to another. With other amino acids, the quantities found necessary vary as much as 100 per cent in different individuals. Moreover, no correlation appears to exist between the requirement on the one hand and the body weight, body surface, or creatinine output on the other. Whether such data indicate that certain essential amino acids can be synthesized in part, but with varying success by different subjects, or whether they merely reflect the extent to which alimentary microorganisms participate in the syntheses, cannot be stated at the present time. Experiments in rats appear to demonstrate that alimentary microorganisms play a much less dominant role in the formation of amino acids than they do in the synthesis of vitamins (10). Regardless of the explanation, the variations in individual requirements serve to demonstrate the tentative nature of the quantitative findings. This fact has always been emphasized when reference has been made by us to any part of these studies. Futhermore, when several subjects show different requirements for a given amino acid, the highest level must be taken as the minimum. Any 'recommended intakes' must make further allowances for the possibility that certain individuals may need still larger quantities than the subjects thus far tested. This aspect of the problem will be considered below.

Perhaps the most disturbing, and certainly the most surprising, feature of experiments involving the use of diets containing mixtures of amino acids relates to the caloric requirements of the subjects. It has been our experience that with such diets the caloric intakes must be increased above the expected levels in order to maintain nitrogen balance. This is true even when the amino acids furnish 7 or more grams of nitrogen daily. We had assumed that intakes of 40 calories per kilo of body weight per day would be sufficient. This, however, proved not to be the case. It seemed necessary, therefore, to compare the caloric needs of individuals, under otherwise identical conditions, when they were ingesting diets containing respectively casein, hydrolyzed casein, and mixtures of pure amino acids as sources of nitrogen. One of 3 such experiments is summarized in table 2.

Throughout the test the daily nitrogen intake was kept within a range of 10.03 and 10.08 gm., and the ratio of calories from carbohydrates to calories from fats was maintained at 2.6. The diets were similar in composition to those employed throughout the quantitative investigations. The acid-hydrolyzed casein was supplemented with L-tryptophan. The mixture of 9 amino acids was composed of the 8 essentials with sufficient added glycine and urea[2] to raise the nitrogen content to the desired level. Hence, the only variables were the sources of nitrogen and the fuel value of the rations.

TABLE 2. CALORIC REQUIREMENTS ON DIETS CONTAINING PROTEINS OR AMINO ACIDS

Period averages *Subject: W. A. S., 71.7 kilos*

SOURCE OF DIETARY N	DURATION OF PERIOD	DAILY INTAKE OF CALORIES	AVERAGE DAILY N BALANCE
	days	*per k.b.w.*	*gm.*
Whole casein	6	45	+0.48
	7	35	+0.14
Acid-hydrolyzed casein	8	35	−0.29
	6	45	+0.50
9 Amino acids	5	45	+0.33
	6	35	−0.91
20 Amino acids	6	35	−0.93
Whole casein	5	35	+0.46
Enzymatically hydrolyzed casein	6	35	−0.09

The subject came into positive balance promptly upon the casein diet when the latter furnished 45 calories per kilo of body weight per day, and continued in positive balance during the 7 days in which the fuel value of the food was reduced to 35 calories per kilo of body weight.

[2] As pointed out later in this paper, extensive investigations in this laboratory have shown that glycine and urea can serve, in the growing rat, as sources of nitrogen for the synthesis of the nonessential amino acids. This is true even to a greater extent of ammoniun citrate.

However, when the whole casein was replaced by acid-hydrolyzed casein supplemented with L-tryptophan, negative balance ensued until the caloric intake was raised to the higher level. Like results were observed with the mixtures of pure amino acids. With the 35 calorie intake, neither the mixture of 9 amino acids (the 8 essentials plus glycine and urea) nor the mixture of all known protein components including citrulline (but without urea) was capable of inducing nitrogen equilibrium. Furthermore, no difference could be detected in the nutritive quality of the two mixtures for maintenance purposes. Several months later, the same subject was used in a comparison of whole casein and enzymatically hydrolyzed casein, as recorded in the last two lines of table 2. As will be observed, he was brought into strong positive balance upon the casein diet with a fuel intake of 35 calories per kilo of body weight. Replacement of the whole casein by the enzymatically hydrolyzed product led to a slight negative balance. Apparently, even the latter may be somewhat inferior to the whole protein for maintenance purposes.

No explanation is apparent for the above observations. Whether proteins contain, or carry as a contaminant, some component which is involved in the efficient utilization of food energy or amino acids and is destroyed by hydrolysis, or whether our findings are attributable to the more rapid absorption of free amino acids, as contrasted with native proteins which are slowly digested in the alimentary tract, is not clear. If the rate of absorption were the critical factor, one might anticipate a considerable loss of amino acids in the urine. Our experience does not warrant that conclusion. Possibly the casein, which was a commercial 'vitamin-free' product, may have carried an adsorbed agent which was not present in the liver concentrate used as a source of the less well-defined vitamins. Tests will be undertaken shortly to determine whether vitamin B_{12} influences the caloric requirements of our subjects.[3]

Since the above comparisons were made, the practice has been followed in the quantitative experiments of administering 55 calories per kilo of body weight per day. Lower intakes might have sufficed in some instances. However, the higher values avoided prolonged adjustment periods. Generally, balance was established within three or four days. Occasionally, somewhat larger intakes were found necessary. This was true of tall, slender individuals who were underweight for their height. One subject who was receiving 55 calories per kilo complained of hunger, and was permitted to increase the fuel value of his ration to 58 calories per kilo.

One may be warranted in asking whether the high energy content of the diets influenced the outcome of the investigations. To this question a categorical answer cannot be given. The necessity of using diets of higher than normal caloric content is demonstrated by the data in table 2, and by additional data of a similar sort not included in the present paper. Furthermore, any criticism of the techniques used in the present studies applies, with equal force, to like tests made with completely hydrolyzed proteins. As has been shown, the latter are not superior to mixtures of purified amino acids as regards the required fuel value of the diets, and have the disadvantage that one cannot be sure, in most instances, of the quantitative removal of the amino acid under investigation. Thus, one is forced to employ high caloric rations if studies of this general nature are to be pursued successfully. Despite the well known protein-sparing action of carbohydrates, it seems unlikely that the minimum requirements of the organism for individual amino acids could have been lowered significantly by the imposed experimental conditions. Even if one assumes that native proteins ordinarily carry an 'accessory factor' the function of which is to promote physiological economy in the utilization of food energy or amino acids, the absence of this factor would be expected to lead to a higher rather than a lower minimum requirement for a dietary essential. It does not seem reasonable to postulate that the absence from the ration of so important a substance, if it exists, would actually lessen the need of the body for something which it cannot manufacture.

Finally, attention should be called to the fact that the experience of others with low protein diets, as contrasted with diets containing amino acids, has led to the use of relatively high caloric intakes. In describing an experiment in which egg protein furnished approximately 95 per cent of the nitrogen, Murlin, *et al.* (11) state that the total energy value at the beginning of the test was placed at 45 calories per kilo of body

[3] There are reasons for suspecting that in the preparation of our liver concentrates from Wilson's 'liver powder 1–20' much of the vitamin B_{12} may have been lost.

weight, "but when in individual cases it became apparent that this allowance was not sustaining body weight it was raised to 48 cal. per kilo or sometimes higher, by addition principally of carbohydrate." Comparable energy intakes were employed by Bricker, *et al.* (12) in a recent study of the protein requirements of young women. One of the 10 subjects needed 50 calories per kilo to maintain body weight. Our young men were encouraged to avoid unusual physical exertion during the course of the tests. However, as graduate students they found it necessary to work long hours in the laboratory, for the most part in a standing position. Also, they walked to and from their living quarters which frequently were located more than a mile from the campus. In the light of these facts, and the strange increase in fuel requirements associated with amino acid diets, the caloric intakes herein employed were not so excessive as first impressions might lead one to believe.

The intricacies involved in a study of the minimum amino acid requirements have been dealt with at some length in order that the reader may be fully advised as to the exact conditions under which the findings described below were obtained. The nitrogen balance technique has its limitations, but, at the present time, it is the only procedure which can be applied to adult man for the purposes in question. Possibly, the preceding pages have over-emphasized the difficulties and imperfections. If so, this is to be preferred, in our judgment, to unwarranted simplification of a problem which is beset by many meagerly understood variables. As Mary Swartz Rose and MacLeod (13) remarked many years ago, "the search for an absolute biological constant in protein metabolism is a vain one; any so-called biological minimum is such only for a given set of conditions."

Despite what has gone before, the values obtained in the present studies are believed to approximate very closely the true minimum needs of the human species. Under the conditions specified, remarkably definite conclusions can be reached regarding the requirements of a given individual for any essential amino acid. A valid criticism of an investigation of this nature is the comparatively small number of tests that can be made in a reasonable time. Thirty-one quantitative experiments have now been completed. These represent a tremendous undertaking, extending over a period of almost four years. The data are presented with complete confidence, but must be viewed as strictly tentative until experience demonstrates their general applicability to a much larger number of individuals.

The procedures followed in the conduct of the quantitative experiments were almost identical with those used in the qualitative tests. One difference is to be noted. In determining which amino acids are necessary dietary components, the removal of one from the food was compensated for by raising all others sufficiently to maintain the nitrogen intake at a constant level. In demonstrating the minimum requirement of the organism for a given component, all amino acids were kept at constant amounts throughout the test except the one under investigation. The latter was progressively lowered, and the nitrogen content of the ration was maintained by the addition of glycine, urea, or both. Growth studies in this laboratory (14) have shown that glycine and urea can be utilized by rats as sources of nitrogen for the manufacture of the non-essential amino acids. The two compounds were included in the human diets for a like purpose. Throughout the entire investigation, the rations furnished approximately 10 gm. of nitrogen daily except during the first two or three tests of the series. In these, the food supplied 7 gm. of nitrogen daily. Thus, an abundance of nitrogen was always available to the cells for synthetic uses. This is an important point, for it does not follow that nitrogen balance could be maintained with the minimum levels of the 8 essentials alone. Under the latter circumstances, the manufacture of the non-essentials by the tissues would be impossible.

One other detail of the procedure should be described. After the minimum requirement for each amino acid had been determined, twice this value was selected as the intake of the subjects in all subsequent experiments involving other amino acids. This may be rendered more intelligible by a specific illustration. The first amino acid to be investigated was tryptophan. The diet employed at the beginning of these tests supplied 1.85 gm. of L-tryptophan daily. By progressively reducing the intake, the minimum level compatible with consistent positive balance was found to be 0.15 gm. daily in each of two subjects. Inasmuch as the two individuals yielded identical results, it was assumed tentatively, and in spite of the remarkable low figure, that 0.15 gm. represented the minimum requirement. Later events have necessitated an up-

ward revision of this value, as will be explained later.

In the meantime, the question arose as to the quantity of L-tryptophan which should be included in the food in measuring the requirements of man for other amino acids. It seemed likely that twice the minimum would be a safe intake. Thus, 0.3 gm. of L-tryptophan per day has been the standard in subsequent quantitative experiments, although in some tests 0.2 gm. was employed successfully. A similar procedure has been followed with each amino acid as its minimum was established. Consequently, the quantitative data have been supplemented by the accumulation of evidence, in a considerable number of individuals, that twice the minima are satisfactory intakes. The latter are arbitrarily being designated as the 'recommended intakes.'

Returning for a moment to the question of the tryptophan requirement, attention is called to the fact that 31 individuals have been kept in positive nitrogen balance upon daily intakes of 0.3 gm. or less. On the other hand, a recent subject has shown a minimum requirement of 0.25 gm. daily. This is decidedly the highest requirement for this amino acid we have ever observed. Eleven other subjects have been kept in balance with intakes of 0.2 gm. daily, although no attempt was made to establish their minimum requirements. Because of the single high value, it seems necessary to regard 0.5 gm. (twice the highest minimum) as the recommended daily intake until and unless later events demonstrate that this amount is unnecessarily large.

In table 3 are summarized the minimum daily requirements and the 'recommended daily intakes' of the 8 amino acids which are necessary dietary components for the maintenance of nitrogen equilibrium in normal human subjects. Both groups of values must be regarded as strictly tentative. In the column to the extreme right is listed the number of subjects in whom each recommended intake has been shown to be satisfactory. As will be observed, the numbers are smallest for leucine and isoleucine, since these amino acids were the last for which minimum values were established.

Many tests have been made to determine the availability in the human organism of the D amino acids. The nitrogen balance technique is not sufficiently delicate to enable one to exclude the possiblity that certain D amino acids may be utilized in trace amounts which are insufficient to affect the balance. On the other hand, one can readily decide whether the D amino acids possess any practical significance in metabolism. Our data show that D- and L-methionine are equally effective in the maintenance of nitrogen equilibrium in man, and that D-phenylalanine is partially utilized although the human organism appears to be limited as to the amount of this amino acid which it can invert. Contrary to its behavior in the rat, D-tryptophan exerts no detectable influence upon nitrogen balance in man. In our experience, this is true also of acetyl-D-tryptophan despite the report to the contrary in the literature (15). The D modifications of valine, leucine, isoleucine, threonine, and lysine appear not to be available at all in human subjects.

TABLE 3. MINIMUM AND RECOMMENDED INTAKES FOR NORMAL MAN WHEN DIET FURNISHES SUFFICIENT NITROGEN FOR SYNTHESIS OF NON-ESSENTIALS (STRICTLY TENTATIVE VALUES)

AMINO ACID	MINIMUM DAILY REQUIREMENT	RECOMMENDED DAILY INTAKE	SUBJECTS TESTED
	gm.	*gm.*	*no.*
L-Tryptophan	0.25	0.5	31[1]
L-Phenylalanine	1.10	2.2	22
L-Lysine	0.80	1.6	27
L-Threonine	0.50	1.0	19
L-Valine	0.80	1.6	23
L-Methionine	1.10	2.2	13
L-Leucine	1.10	2.2	8
L-Isoleucine	0.70	1.4	8

[1] All of these subjects have been kept in balance on 0.3 gm. or less.

Finally, the reader is reminded that *in the formulation of diets of low amino acid content the so-called recommended intakes, rather than the minima, should now be employed.* The purpose of the former is to make allowance for any further possible variation upward in the minimum requirements of individual subjects. Such diets, to be successful, must furnish most certainly sufficient nitrogen for the synthesis of the non-essentials. This point is emphasized repeatedly in this paper because of its utmost importance. No attempt has yet been made to ascertain the minimum total nitrogen content of a diet, carrying the recommended intakes of the eight essentials, which will sustain nitrogen equilibrium. Experiments of this nature will be undertaken at an early date. The results should provide an

approximate measure of the extent to which synthetic reactions involving nitrogen occur *in vivo*. It is contemplated also in the near future to investigate the possible replacement of phenylalanine by tyrosine, and of methionine by cystine and/or choline.

As to the applications of the above findings, it may be stated that the values summarized in table 3, if typical of male adults as we believe they are, should render it practicable, for the first time, to evaluate proteins in terms of their ability to meet human needs. Hitherto, all such appraisals of proteins have been based upon animal tests. Qualitative and quantitative differences are now recognized in the amino acid requirements of man and animals. Consequently, it does not follow necessarily that estimates of nutritive quality based upon animal tests are applicable to human subjects. It should be possible, by reference to the values now made available, to predict with reasonable accuracy how much of a given protein, if its composition is known, will be required to maintain nitrogen equilibrium in the human organism, provided a sufficient supply of nitrogen to cover the synthetic needs of the cells is also included in the food. It is to be hoped that studies of this nature will be undertaken in many laboratories. In this way only can it be ascertained whether the recommended intakes herein proposed possess general applicability. Obviously, tests of this character must take into account the digestability of the protein under investigation.

Another application relates to preparations for parenteral use. Much interest has been manifested in recent years concerning the possibility of successful intravenous alimentation in patients who temporarily are unable to consume food in the usual way. The practicability of such a procedure was suggested by the author almost 15 years ago (16), and since then has been explored in many laboratories. Much of the enthusiasm previously displayed has now vanished. This has been due in part to the adverse clinical reactions which sometimes follow the adoption of this therapeutic measure, and in part to the fact that a relatively large quantity of a protein hydrolysate must be administered in order to approach nitrogen balance. In this connection, it should be realized that the most appropriate mixture for clinical purposes has not yet been prepared, since the prerequisite information respecting the amino acid requirements of man has not hitherto been available. With the presentation of the facts recorded in this paper it should be feasible to formulate a mixture the composition of which is adjusted to the needs of the body for each component. Such a material may prove to be the most efficient source of nitrogen yet devised for parenteral use. One disadvantage which cannot be overcome immediately, and which may have contributed to the discouraging results observed in certain quarters, is the excessive caloric requirement manifested by subjects receiving mixtures of amino acids in place of proteins. Further studies may reveal a method, or a chemical substance, which is capable of counteracting this peculiar feature of amino acid mixtures.

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CHEMICAL MEASUREMENTS IN RELATION TO PHYSICAL EVIDENCE OF MALNUTRITION

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As the science of nutrition has progressed, numerous methods have been evolved for the appraisal of human nutritional status. Some years ago, recognition of malnutrition depended on simple observations, such as gross evidence of loss of weight or dehydration. The demonstration that certain physical stigmata appeared when sufficient quantities of particular chemical substances were not available to the organism, led to refinement and greater specificity of diagnosis. Advances in biochemistry made possible measurement of the concentration of essential nutritive factors and their metabolites in bodily tissues and fluids. Recently, attempts have been made to delineate chemical and physical findings at various levels of nutriture in order to detect incipient malnutrition and to evaluate nutritional status.

In discussing the relationships of chemical tests to physical signs of malnutrition, it is pertinent to emphasize certain concepts of the development of nutritional deficiency. Good nutrition is associated with an abundant supply of all essential nutrients. As malnutrition develops, bodily stores of these factors are depleted, smaller quantities are available to the tissues, urinary excretion diminishes and concentration in the blood falls. Finally a critical level is reached below which metabolic defects occur and impairment of function becomes evident. As the end stage of malnutrition, cellular structure is modified and gross evidence of deficiency disease is apparent. Incipient malnutrition exists when a decrease in reserve functional capacity occurs. Chemical procedures are particularly useful in detecting early abnormalities but are applicable in all zones of nutriture. Physical stigmata occur in chronic advanced malnutrition and may persist as scars long after tissue repletion has been accomplished.

The manifestations of acute and chronic nutritive failure are somewhat different. Acute depletion causes sudden, profound biochemical and physiologic abnormalities, chronic depletion gradual chemical and structural changes, with good compensation for long periods of time. Accurate diagnosis is difficult. Physical signs of malnutrition are non-specific; absence of any one of several factors may give rise to the same structural alteration, while similar tissue change may result from disease unrelated to nutritive failure. A number of the physical signs used as indices of malnutrition rest on a very tenuous foundation. Interpretation should be cautious, since normal variability is great and the question of what constitutes a departure from normal has not been settled.

The concentration of essential nutrients in blood, urine, or other bodily fluids, reflects, in general, recent dietary intake, but pathologic conditions not primarily related to nutrient supply may influence the findings. Load tests, in which a large quantity of a nutrient is administered and changes in the concentration in the blood and urine are measured, are useful in determining the degree of saturation or depletion of the tissues. Saturation, in the sense of maximal tissue concentration, indicates at least adequate nutrition, while unsaturation is a requisite in the diagnosis of malnutrition, although not synonymous with it. Findings in load tests may be misleading in the presence of extensive breakdown of tissue since release of certain nutrients may result in an increase in excretion compatible with saturation. In persons who have suffered long periods of depletion, recent access to nutrients may elevate the concentration in the blood or tissues, without restoring function or reversing pathologic changes. Few chemical or physiologic tests are available for detection of metabolic abnormalities or decreased functional reserve capacity, yet it is here that the early and readily reversible types of malnutrition should be diagnosed. Tests of metabolic derangement are often non-specific and similar abnormalities may occur from causes unrelated to nutrient supply.

The above concepts of the pathogenesis and complexities of malnutrition make it obvious that correlation between chemical measurements and physical findings will not necessarily be observed. Such lack of correlation is common, as will be pointed out in the subsequent discussion of the relationship of chemical to physical findings in specific types of malnutrition.

CALORIC DEFICIT

Caloric deficit is manifested chiefly by physical findings. In childhood, growth failure occurs; in adults, loss of weight, wasting of tissues, weakness and fatigability are the usual findings. Prolonged, severe starvation is manifested by apathy, lethargy, syncope, dry staring hair, dullness of eyes, bradycardia, hypotension, amenorrhea, sterility, and dry inelastic skin with areas of pigmentation (1). Protein deficiency is usually an associated finding and, in any event, the nitrogen balance is negative. Anemia is common, and a low-fasting blood sugar with a diminished tolerance for glucose may be present. A decrease in the basal metabolic rate occurs. Diagnosis of profound caloric deficiency is relatively simple and the severity may be judged by the percentage loss in body weight, the rapidity of such loss, and associated findings of starvation. Detection of mild caloric undernutrition is difficult. There are no adequate standards for weight in relation to height, age, bodily build and genetic potentiality. Simple procedures which will detect the extent of depletion of vital bodily tissues, as compared to fat stores, are not available.

ANEMIA

Anemia is a useful index of malnutrition in spite of its nonspecificity, and determination of the hemoglobin concentration in the blood is widely used in evaluating nutritional status. Anemia may be due to a deficiency of protein, iron or of vitamins of the B complex, notably folic acid and vitamin B_{12}. It is likewise caused by many conditions of non-nutritional origin, such as acute or chronic blood loss, infections and neoplasia. Physical evidence of chronic anemia is minimal in the early stages and diagnosis is dependent on chemical examination. Determination of the size of the red cells (mean corpuscular volume) and of the concentration of hemoglobin in the cells (mean corpuscular hemoglobin concentration) will permit differentiation of the macrocytic anemias, which are the rule in deficiency of protein, folic acid and vitamin B_{12}, from the normocytic or microcytic, hypochromic anemias of iron deficiency. Physical examination aids in detecting non-nutritional causes of anemia and assists in evaluation of its severity. In profound anemia of long standing clinical signs include pallor, glossitis, dyspnea, palpitation, tachycardia, weakness, dizziness, syncope and edema.

Determination of the level of serum iron is a useful procedure in detecting anemia due to iron deficiency. In normal persons on adequate diets, the concentration of serum iron is usually greater than 70 μg. per cent (2–4). Although low levels may occur in acute and chronic infections (5, 6) a concentration of less than 60 μg. per cent is suggestive of iron deficiency (7). Glossitis occurs in iron deficiency anemia (8, 9) as it does in anemia due to deficiency of vitamins of the B complex, and cheilosis has been reported. These physical findings do not correlate with the type or degree of anemia but are more common in anemia of considerable chronicity.

PROTEIN DEFICIENCY

Manifestations of protein deficiency include cessation or slowing of growth, loss of weight, muscle wasting, and edema. Nitrogen balance is negative, serum proteins decrease, particularly serum albumin, and anemia may occur. The concentration of plasma proteins may remain normal for long periods of time in the presence of protein deficiency. This is due in part to hemoconcentration (10) and if the volume of the circulating plasma is determined, protein deficiency may be detected at an earlier stage. Investigations of Elman and others (11) which indicate that a decrease of one gram in total circulating serum protein represents a loss of 30 gm. of tissue protein, suggest that protein deficiency is advanced before a significant drop in the concentration of serum protein occurs.

Correlation has not always been demonstrated between clinical edema and the concentration of albumin or total proteins in the serum. This is not surprising in view of the many factors which influence edema formation. During World War II mild hypoproteinemia was common in certain famine areas (12) and concentration camps (13) but close correlation between edema and the level of serum proteins was not observed. Keys *et al.* failed to obtain such a correlation in studies of 34 men maintained on a European type of famine diet. Govaerts (14), who studied famine edema

in Brussels, found serum proteins to be less than normal in the majority of patients, the mean reduction being 29 per cent. The mean osmotic pressure of the serums was reduced to an even greater extent, namely, 41 per cent. Petrides (15) reported low serum protein values in children with hunger edema in Athens. Denz (16) found a significantly lower concentration of plasma proteins in German civilian internees than in normal British subjects. The degree of edema appeared to be proportional to the reduction in plasma protein concentration but considerable variation occurred. Values for plasma albumin were low (2.58 to 3.81 gm. per 100 cc. of plasma), but were not correlated with the degree of edema. Worsham (17) studied the relationship of hypoproteinemia to edema in women during pregnancy. Edema was present in 8 per cent of women when the serum protein level was above 6.0 gm. per 100 cc., in 21 per cent when the level was between 5.5 to 6.0 gm., and in 72 per cent when the level was less than 5.5 gm.

Further studies of protein nutrition may include measurement of the excretion of individual amino acids. This may be informative as to the biologic value of dietary protein (18), as well as uncovering specific amino acid deficiency.

VITAMIN A

Chemical tests for evaluation of vitamin A nutrition include determination of the levels of vitamin A and carotene in the blood, tests of absorption of vitamin A from the intestinal tract and estimation of the vitamin A content of the liver in specimens obtained by biopsy. Physical findings in vitamin A deficiency are dryness and scaling of the skin, follicular hyperkeratosis, xerophthalmia, Bitots spots, and night blindness or lesser degrees of abnormal dark adaptation.

The normal range of values for vitamin A and carotene in the plasma has not been entirely settled. It has been suggested that the lower limit of normal be placed between 50 and 100 μg. per cent for carotene, and between 40 and 75 International Units (19–22) or 20 to 30 μg. per 100 cc. (23, 24) for vitamin A. Levels of vitamin A in the blood increase with age and seasonal variations have been noted (25). When the intake of vitamin A is reduced, the concentration of carotene in the blood falls rapidly, while that of vitamin A decreases slowly. In Hartzler's experiments (26), it took 140 days of a diet low in vitamin A before a reduction in plasma level was observed. When diets high in vitamin A are administered, concentration in the blood may rise before signs of deficiency disappear (27). Hsu (28) found the mean level of vitamin A in the blood to be 56 I.U. in healthy Chinese, 33 I.U. in hospital patients without clinical signs of vitamin A deficiency, and 6 I.U. in patients with ocular or skin lesions attributable to lack of this vitamin. Mean carotene values in the blood in these same groups were 140, 65, and 24 μg. respectively. Van Veen and Postmus (29) found very low levels of blood vitamin A in the Netherlands East Indies, with a high incidence of poor dark adaptation, xerophthalmia and blindness. In several areas mean levels varied from 27 to 77 I.U. in children, and from 49 to 100 I.U. in adults. Xerosis conjunctivae was usually apparent when values of less than 40 I.U. per 100 cc. were encountered. J. H. Jonxis (30) found low to absent vitamin A in the blood of children in Holland. The first clinical finding observed was follicular keratosis while keratomalacia did not occur. In surveys in Tennessee and North Carolina, Anderson and Milam (31) found no correlation between the plasma level of vitamin A and clinical signs considered to be evidence of vitamin A deficiency. Harris and co-workers (32) found lower values for carotene and vitamin A in clinic patients than in normal subjects but the former had no signs of deficiency. Adamson *et al.* (33) in a survey in Newfoundland, found a significant correlation between serum vitamin A levels and excess tissue in the conjunctivae in children aged 6 to 10 years, but not in other age groups. No correlation was observed between other physical signs and the concentration of vitamin A in the serum. Numerous other studies have failed to show any correlation between the level of vitamin A in the blood and thickening of the conjunctivae (34–36).

Krause *et al.* (37) found no difference in th concentration of vitamin A in the blood in children with and without follicular keratosis but carotene values were lower when folliculosis was present.

A lack of correlation between levels of vitamin A in the serum and follicular keratosis was reported in a survey in Newfoundland (36). Nor El Din (38) found an average concentration of vitamin A in the blood of 40 μg. per cent in ordinary hospital patients, 20 μg. per cent in patients with cirrhosis of the liver or with diabetes, 15 μg. per cent in patients with pellagra, and traces only in 27 persons with catarrhal or obstructive jaundice. Night blindness was present in $\frac{1}{4}$ of the patients who had cirrhosis, diabetes or jaundice, in $\frac{1}{10}$ of those with pellagra. Other workers have

shown that plasma vitamin A values are decreased in many types of disease (39, 40). It has been suggested that the levels of carotene and vitamin A in the blood are an expression of liver function (41). In cirrhosis, a low concentration of vitamin A in the blood and in the liver has been reported and the rise in blood values after oral administration of vitamin A is subnormal (42–44). Clinical evidence of deficiency was not always associated with these findings. In sprue, low values for plasma vitamin A and carotene are often obtained, tolerance tests indicate poor absorption (45) and physical signs of deficiency are frequently noted.

Impairment of dark adaptation has been produced in experimental vitamin A deficiency (46, 47) and several studies of the relationship between abnormalities of dark adaptation, the concentration of vitamin A in the blood, and clinical findings have been reported. In a nutrition survey in Tennessee (48) blood values and findings in visual tests indicated a degree of deficiency comparable to that suggested by dietary intake but few signs of vitamin A deficiency were present. Hasson and Khanna (49) noted that abnormal dark adaptation occurred only when plasma vitamin A was low, but not all subjects with low values had defective adaptation. Caveness, Satterfield, and Dann (50) found only slight correlation between biophotometer readings and vitamin A levels in the blood. Bodansky, Lewis and Haig (51), in a study of infants, found that low levels of vitamin A in the plasma were associated with low stores in the liver and poor dark adaptation. They suggested that plasma levels may be a more sensitive index of deficiency than tests of dark adaptation. Pett and LePage (52) observed a relationship between visual tests and blood levels in vitamin A deficiency. In other studies, no correlation has been demonstrated between dark adaptation response and the concentration of vitamin A in the plasma (53, 54).

The above findings indicate that while some relationship exists between levels of plasma vitamin A and carotene, dark adaptation tests, and physical evidence of vitamin A deficiency, wide individual variations occur. The many diseases which affect vitamin A metabolism make evaluation of vitamin A nutrition a difficult problem.

VITAMIN B COMPLEX

Chemical measurements for a number of the vitamins of the B complex have been developed, but only for thiamine, niacin and riboflavin is there sufficient data relating chemical findings to physical evidence of deficiency to justify discussion at this time.

Early signs of endemic vitamin B complex deficiency, and also of experimentally induced deficiency, include non-specific complaints such as decreased appetite, easy fatigability, lack of ambition and nervousness as well as personality disturbances, including irritability, moodiness, depression, vague fears, lassitude and quarrelsomeness (55). In acute experimental deficiency, outstanding manifestations are disinclination to spontaneous activity, and personality alterations of psychoneurotic type, i.e., depression hypochondriasis and hysteria. Long before these symptoms appear, the urinary excretion of the B vitamins has fallen to low levels (56). In one study (57), the development of mental and physical changes correlated well in the individual case with a rise in pyruvate and lactate in the blood, especially in samples taken after exercise.

Nutrition surveys in Newfoundland have always shown a low intake of the B vitamins and, until recently, a high incidence of beriberi. In 1944, members of two survey groups were impressed by the apathy and lack of spontaneity of the adults, the docility and absence of play among the children (33, 58). In re-surveys in 1948, these findings had disappeared; adults were alert, children were curious, played spontaneously and required monitoring during examination (36, 59). In the interval between 1944 and 1948 the dietary supply of the B vitamins had increased. The urinary excretion of thiamine and riboflavin was much higher in 1948, than in 1944, corroborating dietary and clinical findings.

THIAMINE

Chemical tests used in evaluating thiamine nutrition have included measurement of thiamine excretion in the urine during fasting, for 24-hour periods or for several hours after the administration of an oral or parenteral test dose of this vitamin, determination of thiamine in the blood or in tissues obtained by biopsy, estimation of cocarboxylase in the blood, determination of blood pyruvic acid and of the lactate-pyruvate ratio during fasting, after exercise or following the administration of glucose.

Clinical evidence of thiamine deficiency includes polyneuritis (dry beriberi), cardiovascular disturbances and edema. In severe deficiency acute cardiac failure may occur. Anorexia is common, while neurasthenic symptoms, digestive

disturbances and constipation have been reported as early manifestations.

The urinary excretion of thiamine falls rapidly when the dietary intake of this vitamin is decreased (60–67) and approaches zero when the intake is about 0.07 mg. per day. Mickelsen, Caster and Keys (68) have shown that thiamine excretion is characteristic of the individual as well as being linearly related to the intake, and that excretion of pyrimin, a pyrimidine-like component of the thiamine molecule, is valuable in assaying thiamine nutrition when the dietary supply of this vitamin is low.

The urinary excretion of thiamine during fasting has been found to correlate with the average dietary intake (64, 69, 70, 71) and with the 24-hour excretion (71, 72). This determination has been suggested as a useful procedure in detecting the presence, although not the degree, of thiamine deficiency. It has been suggested that an output of zero to 4 μg. in one hour during fasting is indicative of inadequate thiamine intake or chemical unsaturation (71, 73, 74). Melnick and Field found a lower fasting excretion in subjects with clinical signs of thiamine deficiency than in normal persons. Salcedo and co-workers (75), found that the fasting one-hour urinary excretion of thiamine in beriberi varied from 0 to 8.51 μg. Mean excretion was 2.85, 2.82, and 2.75 μg. in frank, suggestive and doubtful cases respectively. In apparently normal subjects, excretion ranged from 0.7 to 20 μg with a mean of 5.72 ± 3.72. The presence of symptoms and signs of deficiency, together with an excretion of 0 to 2 μg. of thiamine, was considered helpful in the diagnosis of beriberi. Hou (76) found no thiamine in the urine in acute or prolonged, chronic beriberi, a low excretion (14 μg. in 24 hours) in subacute or chronic beriberi of short duration. Excretion in normal persons varied from 17 to 176 μg. in 24 hours.

A number of studies of experimental thiamine deficiency have been carried out. Elsom and co-workers (77) reported that 6 subjects developed clinical signs of thiamine deficiency on a dietary intake of 0.35 mg/1000 calories and excreted less than 42 μg. of thiamine in the urine in 24 hours; 3 subjects on intakes above this level were without signs of deficiency and excreted 40 to 60 μg. Objective signs of deficiency included muscle tenderness and disturbance of vibratory sense. Foresight and judgment, as measured by maze tests, were affected deleteriously. One subject developed edema after 98 days on a deficient diet. The urinary excretion was proportional to the intake and did not change with the appearance of symptoms of deficiency.

Najjar and Holt (62) gradually reduced thiamine intake to 0.128 mg/1000 calories. Urinary excretion had been at minimal levels for many months before this final reduction in diet and no further decrease in excretion occurred when signs of deficiency, i.e., neuritis and edema, appeared. Thiamine load tests, in which the 4-hour urinary excretion was measured after the intravenous injection of 1 mg., showed a decrease preceding and associated with the development of physical evidence of lack of thiamine. Symptoms never occurred when the excretion was more than 60 μg. in 4 hours but were nearly always present when excretion was less than 50 μg. in 4 hours.

Williams and his collaborators (61) found the first chemical evidence of thiamine deficiency to be a decrease in the 24-hour urinary excretion, followed by a decrease in excretion after an intramuscular load test of 1 mg. Almost simultaneously with the drop in test dose excretion, values for pyruvic acid in the blood, after dextrose administration, became elevated, and symptoms of fatigue, listlessness and anorexia appeared. Symptoms progressed to apathy, nausea, epigastric pain, prostration, mental confusion and paresthesias and were associated with a progressive decrease in test dose excretion to less than 100 μg. and a rise in pyruvic acid in the blood. When definite evidence of polyneuropathy appeared, the output following the test dose was less than 50 μg. The load test appeared to be valid for assessment of tissue stores while the level of pyruvic acid in the blood was valid for detecting the biochemical defect of thiamine deficiency, provided other causes of abnormal elevation could be excluded. The biochemical defect developed rapidly when the intake was 0.17 mg/1000 calories, more slowly when dietary thiamine was increased to 0.22 mg/1000 calories, while with an intake of .45 mg/1000 calories, 4 of 5 subjects developed mild metabolic abnormalities and 3 of 5 subjects depletion of tissue stores.

Keys (78, 79) studied healthy adult males on various dietary levels of thiamine and found no change in work output, or in chemical tests related to carbohydrate metabolism, after 10 to 12 weeks on an intake of 0.23 mg/1000 calories, although the urinary excretion of thiamine and the test dose returns were low. When dietary thiamine was reduced to 0.185 mg/1000 calories,

the only evidence of deficiency was an elevation in the resting and post-exercise level of blood pyruvate.

Foltz and associates (68) found a decrease in appetite within 3 weeks on a diet containing 0.33 to 0.38 mg. of thiamine per 1000 calories. Within 4 weeks after the intake was reduced to 0.17 to 0.21 mg/1000 calories, a decrease in work output, an increase in muscle tenderness and pain, fatigue and desire for sleep, and deterioration in mental attitude, nervous stability and alertness occurred. Urinary thiamine excretion ranged from 5 to 20 μg. in 24 hours. No change occurred in blood pyruvic acid values.

Melnick (72) studied normal subjects and persons with clinical symptoms of thiamine deficiency by means of 4 different tests: 24-hour basal excretion, fasting 4-hour excretion, response to the oral administration of 5 mg. of thiamine, and 4-hour excretion after parenteral administration of 350 μg. of thiamine per square meter of surface area. Good correlation was obtained between the tests. With the parenteral test dose, all the normal but none of the deficient subjects excreted more than 50 μg. of thiamine in the subsequent 4-hour period. With this same test, Blanchaer and Cameron (80) found low excretions in debilitated subjects regardless of the etiology of their condition, and larger excretions during convalescence or in subjects with minor illness. The incidence of low excretion increased with age.

Ruffin *et al.* (22) reported a significant difference in the excretion of thiamine by normal persons and patients with clinical findings of mild deficiency of the B vitamins, when a test dose of 1 mg. was given intramuscularly and excretion measured for 4 hours thereafter.

Johnson (73) using a 5 mg. oral load test, concluded that an output of less than 20 μg. in 4 hours was subnormal as judged by nutrition surveys in troops. Goldsmith and Sarett (66), using a similar test, found that normal subjects on adequate diets excreted more than 100 μg. in 4 hours, while persons on diets containing 0.2 to 0.3 mg/1000 calories excreted an average of 58 μg. In patients with clinical evidence of thiamine deficiency, namely, peripheral neuritis or cardiovascular disturbances with edema, the excretion was less than 80 μg. in the 4-hour period.

The thiamine content of blood has been studied by several investigators. Most of the thiamine is found in the cellular elements (81). Foltz (68) reported that the thiamine concentration tends to correspond to the character of the diet, while Greenberg and Rinehart (82) found it to be relatively static and not easily affected by thiamine intake. Thiamine content of the leucocytes has been reported to be decreased in thiamine deficiency (83). Estimation of cocarboxylase in whole blood has been considered an unreliable method of detecting thiamine deficiency (84), but further investigation of this test seems warranted. Oldham *et al.* (64) found a decrease in blood thiamine when the dietary intake was .14 mg/1000 calories. The urinary excretion during fasting and in 24 hours was low, as was the excretion after an oral test dose of thiamine. No definite signs of deficiency occurred after 59 days on this diet but 4 of 9 subjects noted severe constipation. When the intake was raised to 0.2 mg/1000 calories and maintained at this level for 47 days, blood values returned to those found prior to the experiment, as did values for excretion during fasting. However, test dose returns were further reduced, constipation increased, and certain psychologic changes developed, namely, difficulty in grasping ideas and in the ability to concentrate. These symptoms improved when the thiamine intake was raised to 0.36 mg/1000 calories; while urinary excretion increased slightly and test dose returns remained low.

Hulse (85) reported a decrease in the yeast stimulating activity of plasma and in the thiamine content of muscle in subjects maintained for 18 days on a diet containing 0.3 mg. of thiamine per 1000 calories. The only clinical finding was a vague loss in 'sense of well being.' The relationship of blood levels of thiamine to definite clinical signs of deficiency (beriberi) has not been reported. Blanchaer and Cameron (80) found a significant negative correlation between blood cellular thiamine and urinary excretion of less than 90 μg. following a load test of 350 μg. per square meter of body surface. Defective renal clearance could produce such findings, which suggests caution in classifying subjects as normal or deficient solely on the basis of response to load tests.

As noted above, elevation of pyruvic acid in the blood occurs in thiamine deficiency. Since numerous other conditions can lead to a similar rise in pyruvic acid, Allibone (86) has suggested that a decrease in the level of pyruvic acid after administration of thiamine may serve as a test of 'subclinical' deficiency. Investigation by Stotz and Bessey (87) indicated that determination of the lactate-pyruvate ratio might be of assistance

in evaluating thiamine nutrition. Goldsmith (88) found a change in this ratio in patients with polyneuropathy and heart disease due to thiamine deficiency, as well as in persons with physical signs suggesting deficiency of other vitamins of the B complex. Horwitt (89) has suggested that simultaneous measurement of lactic acid, pyruvic acid and glucose in the blood, after mild exercise and glucose ingestion, may prove to be a valuable procedure in detecting early thiamine deficiency. When thiamine intake was 0.2 mg. daily, an apparently pathologic level was observed with this test one to four months before 'incontrovertible clinical signs of thiamine deficiency appeared.'

From the many studies of thiamine nutrition discussed above, it seems clear that 2 chemical procedures have particular merit in detecting thiamine deficiency: *1*) the test suggested by Horwitt (89) in which the levels of glucose, pyruvic acid and lactic acid in the blood are measured after a metabolic load is placed on the organism, and *2*) measurement of the urinary excretion after an oral or parenteral test dose of thiamine, which permits estimation of the degree of saturation or depletion of the tissues with this vitamin.

RIBOFLAVIN

Chemical measurements suggested for evaluation of riboflavin nutrition are estimation of the quantity in the blood, either in the serum or cellular elements, and determination of the urinary excretion in 24 hours, in one hour during fasting and for several hours after the oral or parenteral administration of a test dose. Clinical signs ascribed to riboflavin deficiency include corneal vascularization, seborrheic dermatitis, glossitis, cheilosis, angular stomatitis, and lesions of the vulva and scrotum. Each of these signs has been noted in conditions other than riboflavin deficiency and the specificity of the syndrome has been questioned (90). Slight changes in corneal vascularity occur in normal subjects, do not correlate with riboflavin intake and fail to respond to therapy with riboflavin (91). Although vascularizing keratitis may be due to lack of riboflavin, this sign alone is not diagnostically significant. Cheilosis, angular stomatitis and glossitis may occur in deficiency of any one of several essential nutrients and in states unrelated to inadequate nutrition. The uncertainties in clinical diagnosis render interpretation of the relationship of chemical to physical findings most difficult.

Many studies have shown that the urinary excretion of riboflavin parallels dietary intake until high levels are reached (92, 93). Excretion may be dependent in part on the protein content (94) of the diet and a high excretion may occur after several days of fasting (95). In subjects maintained on diets restricted in riboflavin, basal excretion is low as in the response to an oral or parenterally administered test dose (61, 66, 93, 96, 97). In most instances, evidence of tissue depletion has not been associated with clinical findings considered to be characteristic of riboflavin deficiency. Horwitt (89) concluded that attempts to correlate riboflavin excretion with nutritional state were of little value except as indicative of dietary intake. Recent studies have shown that the riboflavin requirement is related to body size rather than to caloric intake (98) which may explain apparently divergent conclusions in metabolic balance experiments.

Sebrell *et al.* (99), who produced cheilosis, angular fissuring and slight seborrheic lesions in patients on a diet containing 0.5 mg. of riboflavin per day, found a mean excretion in 24 hours of 77 μg. with no decrease in output in the presence of objective signs of deficiency. Keys' (100) subjects who received .99 mg. per day showed no clinical evidence of deficiency and no change in work performance after 52 days on this regime. The urinary excretion in the last few months was about 13 per cent of the intake while the test dose excretion, which was reduced after the first week, remained fairly constant, with perhaps slight reduction at the end of the experiment. Williams (101) maintained subjects on 0.7 mg. of riboflavin per day for 288 days. No clinical signs of deficiency developed but the 24-hour excretion of riboflavin was low (14% of the intake) and there was a progressive decrease in the excretion of an intraperitoneal test dose of 2 mg. of sodium riboflavin. Williams' (101) subjects received a greater daily intake of riboflavin than Sebrell's and had been fortified with riboflavin prior to the experimental period. Davis and associates (93) maintained subjects on .54 mg. of riboflavin daily for over 100 days. No clear evidence of riboflavin deficiency developed. Urinary excretion averaged about 80 μg. in 24 hours and 8 μg. in one hour during fasting. Only 4 per cent of a test dose of .02 mg. per kilogram of body weight was recovered in the urine while 20 per cent was recovered in the pre-experimental period. It seems likely that prolonged depletion is necessary for signs of deficiency to become

evident in persons previously well supplied with this vitamin.

No given level of riboflavin excretion can be suggested as indicative of clinical deficiency but when the dietary intake is about 0.5 mg. per day, the level at which deficiency has been demonstrated, from 50 to 100 μg. is the usual output in 24 hours. This level of excretion may be present with or without physical evidence of deficiency, and no further change occurs when lesions develop.

Kark and Bean (102) found urinary riboflavin excretion, in the post-absorptive state, lower in Indian and Occidental troops and reported a higher incidence of folliculosis, corneal invasion and cheilosis in the Indian group. Hou and Dju (103) reported a daily riboflavin excretion of 100 to 200 μg. in normal Chinese, an excretion of 39 μg. in subjects with glossitis and of 27 μg. in patients with ocular lesions of riboflavin deficiency. Anderson *et al.* (104), in studies in Mexico found the mean riboflavin excretion to be 0.398 ± 0.052 mg. per liter in the population as a whole. Persons with angular stomatitis excreted, on an average, 0.186 ± 0.023 mg. per liter.

In studies in Newfoundland, no correlation was demonstrated between clinical signs of riboflavin deficiency and urinary excretion of this vitamin either in random specimens or after a test dose had been administered (33, 36). However the mean excretion following a test dose was lower in subjects in Newfoundland than in apparently normal persons in this country (36). Goldsmith and Sarett (105) found no significant difference between normal subjects and patients with signs of vitamin B complex deficiency in 4-hour urinary excretion after an oral test dose of riboflavin. Occasionally, high excretions occurred which could not be adequately explained.

Najjar and Holt (106) placed infants on a diet devoid of riboflavin and reported a 24-hour urinary excretion of 40–50 μg., a serum riboflavin level of 0, and a reduction in the riboflavin content of the cellular elements of blood. When 0.4 to 0.45 mg. of riboflavin was given daily, the vitamin reappeared in the serum. Burch *et al.* (107) showed that total riboflavin concentration in the serum decreased by 8 to 25 per cent in 4 subjects whose dietary intake of riboflavin was reduced to 0.4 mg. daily for 4 days. Axelrod (108) found essentially the same riboflavin values in the blood in persons with cheilosis and conjunctivitis as in normal subjects. In a survey in Newfoundland, no correlation was demonstrated between the level of riboflavin in the serum and glossitis, cheilosis or circumcorneal injection (36).

It is obvious that the state of saturation or depletion of the bodily tissues can be determined by chemical tests for riboflavin, but that functional and structural impairment can not be diagnosed by these measures.

NIACIN

Chemical tests for evaluation of niacin nutrition have not been very satisfactory. Niacin is excreted in the urine in several forms, and although reliable procedures are available for measuring acid-hydrolyzable niacin and N^1-methylnicotinamide, it has not been possible, until recently, to account for more than 30 to 35 per cent of the quantity of niacin administered. In 1946 Knox and Grossman (109) isolated a compound from urine, N^1-methyl-6-pyridone-3-carboxylamide, which appears to be the major excretory product of niacin. Determination of the excretion of this pyridone should assist in appraising human niacin nutrition. Unfortunately great difficulty has been encountered in developing an accurate and simple procedure for this determination and only 2 reports have been published dealing with this subject. (110, 111) Other chemical tests used in studying niacin nutrition are determination of niacin and of pyridine nucleotides in the blood, particularly in the cellular elements, and of pyridine nucleotides in the tissues (112). There is a paucity of data concerning the concentration of pyridine nucleotides at various levels of nutriture. The few studies available suggest that neither the measurement of coenzymes or of niacin in the blood offers information of diagnostic importance in niacin deficiency (113–116). Nevertheless, further studies along this line may prove valuable.

The clinical diagnosis of mild niacin deficiency also presents certain difficulties. The signs which appear prior to the development of frank pellagra, with characteristic dermatitis, are glossitis, diarrhea, and certain mild psychic disturbances, all of which are non-specific. Diagnostic reliance has been placed largely on lingual abnormalities but deficiency of other B vitamins and conditions unrelated to malnutrition can produce indistinguishable lesions.

Investigation of the urinary excretion of niacin derivatives has shown that the output of freely hydrolyzable niacin varies little with changes in diet and only a slight decrease occurs in pellagra. Early work indicated a decrease in 'trigonelline'

excretion in niacin deficiency, both in a 24-hour period and for several hours after the administration of a test dose of niacinamide, but there was overlapping of findings in normal and deficient subjects (117–119). Subsequently it was shown that the excretory product of niacin was N¹-methylnicotinamide rather than trigonelline, and several procedures were developed for determination of this metabolite. Until a procedure is developed which can be used to measure the excretion of N¹-methyl-6-pyridone-3-carboxylamide the only biochemical measurement which can be applied in assessment of niacin nutrition is estimation of N¹-methylnicotinamide in the urine in 24 hours, during fasting or after a test dose of nicotinamide has been administered. The relationship of findings in these tests to physical evidence of niacin deficiency has been studied in several laboratories. The intricacies of niacin metabolism make interpretation of findings extremely difficult. There is marked individual variation in the percentage of various metabolites of niacin excreted in the urine. The availability of methyl groups in the body may be a factor in this variation. Of perhaps greater importance, is the relationship between tryptophan and niacin metabolism which has been demonstrated in man as well as in experimental animals (120-121). The excretion of N¹-methylnicotinamide (N¹Me) appears related to the tryptophan as well as the niacin content of the diet. The administration of tryptophan to persons on normal or restricted diets leads to an increase in the excretion of N¹-Me and of a niacin compound obtained by hydrolysis with strong acid. Goldsmith and Sarett (122) have shown abnormalities in the urinary excretion of derivatives of both niacin and tryptophan in pellagra. Furthermore, when patients with pellagra were maintained on diets low in niacin and protein and were given tryptophan, improvement in mental symptoms, glossitis and dermatitis resulted. The relationship of diets high in corn to the pathogenesis of pellagra has not been completely elucidated. The low tryptophan content of corn may be the explanation but the presence of some inhibitory substance has not been ruled out.

Studies of niacin nutrition in the future must include consideration of the dietary intake of tryptophan as well as of niacin and measurement of excretion of this amino acid and its metabolites, as well as of the excretion of niacin, N¹-methylnicotinamide and of N¹-methyl-6-pyridone-3-carboxylamide.

The status of chemical tests used currently in determining niacin malnutrition may be briefly summarized: The excretion of N¹-Me in 24 hours usually falls to low levels in patients with pellagra and in subjects maintained on diets low in niacin and protein. (120, 123, 124) Reports of normal excretion of N¹-Me on diets containing minimal quantities of niacin (125) may perhaps be explained by the tryptophan content of the diets. A low or zero excretion of N¹-Me in the urine during fasting is indicative of low niacin stores in most instances. Normal persons, however, occasionally excrete no N¹-Me in the post-absorptive state (126). A high output of N¹Me has been reported in prolonged fasting and in diseases associated with severe muscle wasting. (126, 127)

Normal subjects usually show a prompt rise in N¹-Me excretion after a test dose of nicotinamide (66, 69, 73, 95, 128). A similar rise occurs in persons who have been maintained on diets low in niacin but supplemented with tryptophan (120). Johnson (73) suggested that an excretion of less than 0.5 mg. of N¹-Me, in 4 hours after an oral test dose of 50 mg. of niacinamide, indicated chemical deficiency. With a similar oral test, Goldsmith and Sarett (66, 105) found the mean excretion of N¹-Me to be lower in patients with signs of vitamin B complex deficiency than in normal subjects. Perlzweig *et al.* (95) administered a single dose of 100 to 500 mg. of niacinamide and noted that subjects with signs suggestive of deficiency of the B vitamins excreted only about half as much N¹-Me as did normal subjects. Ruffin *et al.* (22) reported that the urinary excretion of N¹-Me after a test dose of niacinamide was lower in patients with glossitis, papillary atrophy, cheilosis and peripheral neuritis than in normal individuals. The significance of these data is greatly decreased in that many persons who are hospitalized show a low excretion after a test dose of niacinamide and yet have no lesions of deficiency. More precise evaluation of niacin nutrition awaits further investigation.

VITAMIN C

Chemical tests for assessment of vitamin C nutrition include determination of the concentration of ascorbic acid in the plasma, white cell-platelet layer or whole blood, estimation of the urinary excretion during fasting and in a 24-hour period, and load tests in which changes in blood levels and/or urinary excretion are measured for varying periods of time after administration of a dose of vitamin C. Certain changes in these

chemical tests occur before the appearance of physical evidence of scurvy. In an individual whose bodily tissues are saturated with vitamin C, the 24-hour urinary excretion reflects any increase in the amount of this vitamin ingested. The excretion falls when the dietary intake is reduced but is a poor index of deficiency since in the unsaturated person most of the ascorbic acid reaching the kidney is reabsorbed (129). Although 24-hour urine excretion is not a measure of vitamin C deficiency, there is no ascorbic acid in the urine in clinical scurvy.

The plasma concentration of ascorbic acid reflects dietary intake: when 75 to 100 mg. of vitamin C are included in the diet, plasma levels of 1 to 1.4 mg. per 100 cc. are observed; when only 15 to 25 mg. are ingested, values range from 0.1 to 0.3 mg. (130). When vitamin C is removed from the diet, the plasma concentration falls fairly rapidly to zero without any physical evidence of scurvy. The ascorbic acid of the white cell-platelet layer decreases more gradually and is a better index of total body concentration (131, 132). Plasma ascorbic acid levels of zero have been reported in scurvy (133–135), and also in persons without clinical signs of vitamin C deficiency. A number of pathologic states, notably infections are associated with reduction in plasma ascorbic acid concentration.

Load tests measure the degree of saturation of the tissues with ascorbic acid. Ralli and Sherry (129) found that normal subjects excreted 50 per cent of a dose of 100 mg. given intravenously, while scorbutic subjects excreted less than 5 per cent.

Kadji and co-workers (136) concluded that a correlation existed between the level of plasma ascorbic acid 4 hours after the intramuscular injection of 200 mg. of vitamin C and clinical signs of ascorbic acid deficiency in infants. Values below 0.2 mg. per cent were found in scurvy, and below 0.6 mg. per cent when bodily stores were seriously depleted. Clinical evidences of scurvy were swollen, bluish or bleeding gums, sharp costochondral junctions, swollen and tender extremities, and in most instances typical x-ray changes. Numerous other load tests have been described (137–139) in which vitamin C is given orally or parenterally and the urinary excretion or blood concentration determined at intervals thereafter. Oral tests are influenced by rate of absorption from the intestinal tract, intravenous tests by the ability of the kidney to compete with other tissues when the vascular system is flooded with ascorbic acid. A procedure suggested recently for evaluating tissue depletion is administration of an amount of ascorbic acid larger than the expected deficit (1–3 gm.), orally in divided doses throughout the day, with collection of all urine for 24 hours. The quantity of the test dose retained is estimated with allowance for 25 per cent destruction. An absolute figure for tissue depletion is thus obtained (140).

Studies of induced vitamin C deficiency have been made by several groups of investigators. Crandon, Lund and Dill (141) found that plasma ascorbic acid concentration reached zero after 41 days of a vitamin C deficient diet. The white-cell-platelet layer reached zero after 121 days. The first clinical signs of scurvy, perifollicular hyperkeratotic papules over the buttocks and posterior aspects of the thighs, appeared in 132 days. Perifollicular hemorrhages and petechiae on the lower legs were noted after 161 days. No gross changes in the gums occurred, but x-ray films of the teeth showed interruptions of the lamina dura. Capillary fragility tests remained negative. In total ascorbic acid deficiency, experimental wounds healed poorly. At this time, when 1 gm. of ascorbic acid was injected intravenously, the plasma level fell to zero in 5 hours.

In Farmer's (138) experiments, a diet containing 0 to 10 mg. of vitamin C was administered. Plasma ascorbic acid fell to zero in 70 days while the white cell-platelet level did not reach zero until the end of the fifth month. The physical signs of ascorbic acid deficiency were hyperkeratotic papules surrounding hair follicles, poor healing of experimental wounds, and a measurable decrease in work output. No spontaneous bleeding occurred but petechiae were noted in areas of surgical manipulation. No oral pathology was noted grossly, on biomicroscopic or on x-ray examination. Depletion must apparently be more protracted for characteristic changes in the gums or atrophy of alveolar bone with loosening of the teeth to occur. A saturation test, which consisted of measuring the blood concentration and urinary excretion of ascorbic acid at hourly intervals for 5 hours after the administration of 15 mg. of ascorbic acid per kilogram of body weight, proved to be a reliable index of tissue depletion.

Peters *et al.* (142) studied 10 subjects on a diet low in vitamin C; hyperkeratosis occurred in 17 to 21 weeks, exacerbation of acne in 22 weeks, perifollicular hemorrhages and tiny hemorrhages in the tips of the interdental papillae in 26 to 34 weeks, and poor wound healing in 36 weeks. The

gums became grossly abnormal (purplish, swollen and spongy) in 2 subjects. These two were the only ones showing gingivitis and peridontal disease before the experiment. No changes occurred in the capillary resistance test. Plasma ascorbic acid remained below 0.05 mg. per cent after 5 weeks and the concentration in the white cells dropped below 1 mg/100 gm. after 16 weeks. A leukocyte concentration of less than 2 mg/100 gm., on repeated analysis, was suggested as supporting the diagnosis of scurvy.

Johnson *et al.* (143) found no detectable change in physical vigor in manual workers after 2 months on a diet containing no vitamin C although minimal gum changes were observed. Severe desaturation of tissues was shown by chemical measurements, including blood levels, urinary excretion and load tests.

In surveys of nutritional status, attempts have been made to determine the relationship of changes in the gums, capillary fragility, purpura or petechiae, and follicular hyperkeratosis, to vitamin C nutrition. In a number of studies (144–149) little or no correlation has been demonstrated between changes in the gums and the amount of ascorbic acid in the plasma or the degree of saturation of the tissues with vitamin C. Leeson (148) administered supplements of ascorbic acid to patients with low plasma ascorbic acid levels (mean 0.07 mg. per cent) and obtained no change in hyperkeratosis, petechial hemorrhages, joint signs or gingivitis, although a marked rise in plasma ascorbic acid occurred. In a few investigations, the incidence of gingivitis appeared related to the intake of ascorbic acid or to the levels of the vitamin in the plasma (134, 150, 151, 152). Linghorne's (150) careful study showed that ascorbic acid intake influenced significantly the incidence of gum lesions where local treatment of gingivitis preceded the test period. Adamson *et al.* (33) found a definite correlation between serum ascorbic acid and gum changes, particularly acute hyperemia, swelling, pain and bleeding on pressure, the correlation being marked in persons under 20 years of age. It may be noted that in experimental deficiency, changes in the gums do not occur before other evidences of scurvy.

The capillary fragility test is of no value in diagnosing vitamin C deficiency (129). No correlation was demonstrated between capillary fragility and saturation tests by Holland and coworkers (153). Likewise, no correlation was noted between follicular hyperkeratosis and the degree of unsaturation of the tissues with ascorbic acid by Durham (154) or by Goldsmith and associates (36). In a survey in Newfoundland only a slight correlation, of questionable statistical significance, was observed between gum changes (marginal redness, swelling, recession, or atrophy of the interdental papillae) and values for serum ascorbic acid (136).

The studies noted above, and many others have shown a correlation between dietary intake of vitamin C and the concentration in the plasma or the excretion in the urine. Load tests are indicative of the degree of saturation or depletion of the tissues with respect to this vitamin. The concentration of ascorbic acid in the white cell-platelet layer of the blood is an index of tissue stores and is found to be zero prior to the appearance of physical evidence of scurvy. These findings are most useful in the diagnosis of vitamin C malnutrition but it should be emphasized that the presence of infection, burns, or the use of certain drugs, may influence ascorbic acid nutrition including the findings in chemical tests.

VITAMIN D

The chemical evaluation of vitamin D nutrition is dependent on measurement of the levels of alkaline phosphatase, of calcium and of phosphorous in the blood. There are no methods for estimation of vitamin D in materials of low potency. Normal values for alkaline serum phosphatase range from 5 to 15 Bodansky units in infants and young children and from 3 to 5 units in adults (155, 156). It has been suggested that 20 units is the upper limit of normal in premature infants (157). Phosphatase activity increases early in rickets, the rise is related to the severity of the rachitic process, and activity returns to normal when healing occurs (156–158). In mild rickets values of 20 to 30 units have been noted; in severe rickets values as high as 60 units (159) have been observed. In the age group in which rickets is prevalent and easily detected, other causes of an increase in serum phosphatase are uncommon. Klasmer (156) found a parallelism between the severity of clinical signs of rickets, particularly cranio-tabes and swelling of the epiphyses, and serum phosphatase activity. Only rarely was there clinical and x-ray evidence of rickets without elevation of serum phosphatase. Determination of phosphorous in the serum was a less reliable measure of vitamin D deficiency, a reduction being noted only in severe rickets, and at times in the absence of rickets. Barnes and Carpenter (158) reported that roentgen examination and estimation of serum phosphorous failed

to detect early rickets in a large percentage of cases, while good agreement was obtained between the degree of phosphatase activity and the development of clinical rickets or the rate and progress of healing. Sydow (160) found elevated phosphatase values in association with roentgenographic evidence of rickets. The level of serum calcium was unrelated to these findings. Determination of serum calcium is valuable primarily in the differential diagnosis of tetany.

The significance of elevated alkaline phosphatase values in children over 5 years of age has been questioned (161). Bessey (162) found a slight increase in certain groups of school children and suggested that determination of phosphatase activity might be useful in nutrition surveys. In view of the histological evidence of rickets reported by Follis *et al.* (163) in 46 per cent of children who died between the ages of 2 and 14, further studies of serum phosphatase in older children seem indicated. Elevation of serum phosphatase has been reported in osteomalacia.

These and other data indicate that phosphatase activity is the most sensitive index of active rickets, while skeletal abnormalities and roentgenographic changes are also useful in the diagnosis of avitaminosis D.

VITAMIN K

The detection of vitamin K deficiency depends on indirect measurements to determine the amount of prothrombin in the blood. In the absence of vitamin K, hypoprothrombinemia occurs and the blood-clotting time may be greatly prolonged. The clinical manifestation of vitamin K deficiency is hemorrhage. Vitamin K is synthesized by micro-organisms in the intestinal tract and dietary deficiency is extremely rare. Since bile salts are necessary for the absorption of this fat soluble vitamin, deficiency occurs when bile is absent from the gastrointestinal tract. It is also observed in the new born, in steatorrheas, and after the administration of certain drugs. Hypoprothrombinemia occurs in severe liver disease but in this instance is not due to vitamin K deficiency but to disturbed liver function (164). Various methods (165–167) of determining prothrombin in the blood have been proposed. A number of variables other than prothrombin influence findings with all these procedures. Consequently, the exact concentration of prothrombin at which hemorrhagic phenomena occur can not be stated. When prothrombin activity exceeds 20 per cent of normal, hemorrhage is rarely due to deficiency of prothrombin. Studies in persons receiving therapy with dicumarol indicate that bleeding seldom occurs unless prothrombin activity is less than 10 per cent of normal (168, 169). Alexander (170) has reported that a prothrombin concentration of between 5 and 10 per cent of normal, determined by the method of Rosenfield and Tuft (171), is a reasonably safe level.

In the above discussion only the chemical measurements and physical signs commonly used in the appraisal of human nutritional status have been considered. Problems of mineral nutrition and electrolyte balance were of necessity omitted from this brief resume. Chemical tests for some of the newer vitamins have not been sufficiently correlated with physical evidence of malnutrition to be included.

Research up to the present time has shown that chemical tests are useful in reflecting recent dietary intake of a number of nutrients, in determining adequacy of body stores of several dietary factors, and in detecting early functional derangement of a few of these essential substances. The presence of certain physical signs, in conjunction with chemical findings, will permit diagnosis of specific types of malnutrition in many instances. Accurate diagnosis in nutrition, as in other fields of medicine, will always be dependent on careful evaluation of data from many sources and marked specificity of findings can not be anticipated. Future study of chemical and physical changes in various zones of nutriture should provide methods for more precise diagnosis of malnutrition, both in regard to nutrients now known to be important, and to new factors whose role in bodily economy has not yet been clarified.

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ANEMIAS CAUSED PRIMARILY BY MALNUTRITION

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I SHALL not attempt to review the older literature on this subject since I am sure it is familiar to most of this audience. Instead, I shall devote my time to a discussion of current research and the important problems which loom in the immediate future.

Advances in this field have been so rapid that I am no longer entirely sure what my title actually embraces. Research on anemia is constantly dragging nearly all anemias closer to nutrition. This is not too surprising if we think of blood as a body tissue which is constantly growing and being replaced at a rapid rate; also that it is a highly organized tissue of great complexity involved in many intricate and rapid chemical reactions. It therefore *must* be bound intimately with the nutritive factors which it must both transport and utilize.

Unfortunately, the word anemia has been very loosely used to mean a variety of defects.

Blood, being so complex, can be varied in a number of different ways. It is necessary to consider not only alterations in the number of the red blood cells, but also variations in their size, in the content of hemoglobin and in the total quantity of blood. In considering etiology, it is necessary to determine whether the disturbance is due to interference with or cessation of production, a block in maturation, increased destruction, or blood loss.

Without attempting to classify the anemias of malnutrition, they may conveniently be discussed in two groups: *1*) those microcytic or normocytic anemias which accompany nutritional deficiencies, *2*) the macrocytic anemias.

In the category of microcytic or normocytic anemias which accompany vitamin deficiencies, the most important are those associated with the vitamin B complex.

There is no need to discuss each of the B vitamins individually in relation to anemia. Biotin, P-amino benzoic acid and choline seem to be little concerned directly with erythropoiesis; nor does thiamine appear to be of great importance. Riboflavin, pyridoxine, pantothenic acid and niacin are of sufficient interest to warrant some attention.

In view of the enormously complicated chemistry of the blood, it is not surprising that a number of different substances are intimately concerned in its formation and normal function, or that complex interrelationships exist. This is a field in which we are obviously just beginning to learn the fundamentals. As with other symptoms of nutritional difficulty, anemia is a nonspecific end result which may occur when any one of many essential substances for formation is missing.

RIBOFLAVIN

There is much evidence of the importance of riboflavin in erythropoiesis in several species of animals. Several observers have reported anemia in dogs on riboflavin deficient diets (1–3) and have observed favorable responses to treatment with riboflavin (4). It also has been observed in rats (5, 6), swine (7), and monkeys (8, 9). The added strain of hemorrhage has helped to bring out the effect of riboflavin deficiency on erythropoiesis (3, 6). In some instances the anemia seen in riboflavin deficient animals has not responded to the administration of riboflavin (1, 10), suggesting a complex relationship with other factors or the presence of some other unrecognized deficiency. A similar situation was observed by Moore, Minnich, Vilter and Spies (11), who found that the anemia in their patients with riboflavin deficiency responded to iron alone. This usually would be regarded as proof of a concomitant iron deficiency; but the observation of Darby (12, 13) that some patients with angular stomatitis similar to that of riboflavin deficiency respond to iron therapy suggests the possibility of an iron-riboflavin relationship in erythropoiesis.

Riboflavin has, of course, been tried in pernicious anemia without effect (14). It is rather

surprising that in spite of the obvious importance of riboflavin in experimental anemias in animals there is such little evidence of any effect in man.

PYRIDOXINE

Here, as in the case of riboflavin, there is much proof of an effect of pyridoxine on erythropoiesis in experimental animals. It has been observed in dogs (15–17), rats (18, 19), monkeys (20), swine (21), chicks (22), and ducks (23). In rats the added strain of hemorrhages shows the effect more clearly (19), as is the case with riboflavin. One of the very interesting observations that runs through the pyridoxine anemia literature is that once it is produced, complete remission cannot be obtained by pyridoxine unless a liver concentrate is also used (24, 20). This does not appear to be due to folic acid (20) and further strengthens the view, as with riboflavin, that there are complex interrelationships in these anemias. The work of Cartwright, Wintrobe and Humphreys (25) with swine has shown that a combined deficiency of iron and pyridoxine results in a more severe anemia than with deficiency of either alone, and that serum iron is unusually high in pyridoxine deficiency anemia. The administration of pyridoxine results in a rapid fall in plasma iron and a rapid rise in red cell count, indicating that the animal continued to absorb iron although unable to utilize it. This, I am sure, will suggest interesting possibilities for a role of pyridoxine to many members of this audience.

As complex as this possible interrelationship may seem, it is relatively simple compared to the real possibilities. Thus we see that a deficiency of pyridoxine results in a disturbed tryptophane metabolism as indicated by increased excretion of xanthurenic acid and kynurenine (26, 27). This introduces the possibility of a relationship between amino acids and anemia, as well as a role in the well known tryptophane-niacin effect which also is concerned in erythropoiesis, as will be discussed later in this paper.

In spite of all the evidence on animals, just as with riboflavin, there is no proved effect of pyridoxine on erythropoiesis in man (28, 11, 29, 30).

PANTOTHENIC ACID

In the case of pantothenic acid deficiency, as with pyridoxine, there is much evidence of an effect on erythropoiesis in experimental animals, but no good direct evidence that it is concerned in anemia in man.

Anemia in pantothenic-acid-deficient animals has been observed with dogs (31), swine (32), monkeys (20), and rats (33). There is also evidence here, as with pyridoxine, that the problem is not a simple one, in that in some instances complete recovery does not follow the administration of pantothenic acid. Daft *et al.* (33) have obtained a partial explanation for this. Working with pantothenic acid-deficient rats, they found that the pantothenic acid deficiency resulted in a secondary folic acid deficiency. This was not the complete explanation, however, and there is still the likelihood of another unidentified factor being concerned.

The important point is that with pantothenic acid deficiency, as with pyridoxine deficiency, the failure to obtain a complete therapeutic result with the synthetic vitamin after the deficiency has been produced may be interpreted as indicating a probable interaction or metabolic relationship with other factors essential in erythropoiesis.

NIACIN

The complexities in the possible role of niacin in erythropoiesis make this substance especially interesting. In the case of niacin, effects have been observed both in man (34) and in experimental animals (35–37). However, the observations in man are so complicated by multiple deficiencies that no clearcut human anemia due to niacin can be recognized at this time (38). In the case of swine, the observation of Petri *et al.* (39) is of special interest. These workers found that removal of the fundus of the stomach of swine led to a loss of the anti-anemia substance from the liver, which could be prevented by nicotinic acid. This observation is of such importance, if true, that the work should be confirmed and extended.

Wintrobe *et al.* (40) have found that niacin deficiency in swine does not develop unless the diet is also poor in protein. The anemia can be corrected either by protein or niacin.

The work with niacin deficiency anemia in dogs is of especial interest (41). Handler and Featherston (42) observed anemia in dogs on three different diets deficient in niacin. They postulate that the effect of niacin deficiency is through deficiency in cozymase since niacin functions as a constituent of pyridine nucleotides.

One interesting aspect of niacin deficiency anemia in dogs is based on the observation of Rhoads

et al. (43) 11 years ago, that on the Goldberger diet dogs were more sensitive to an anemia produced by feeding indole than were dogs on normal diets, and that this extra hemolytic effect of indole under these conditions could be abolished by liver extract. Sebrell and Hundley (44) have recently reopened this subject with some interesting results.

We have been able to show that dogs on the Goldberger diet number 123 given 500 mg. of indole daily develop a severe anemia which can be corrected by the administration of 100 mg. of niacinamide daily, in spite of continued administration of indole (fig. 1).

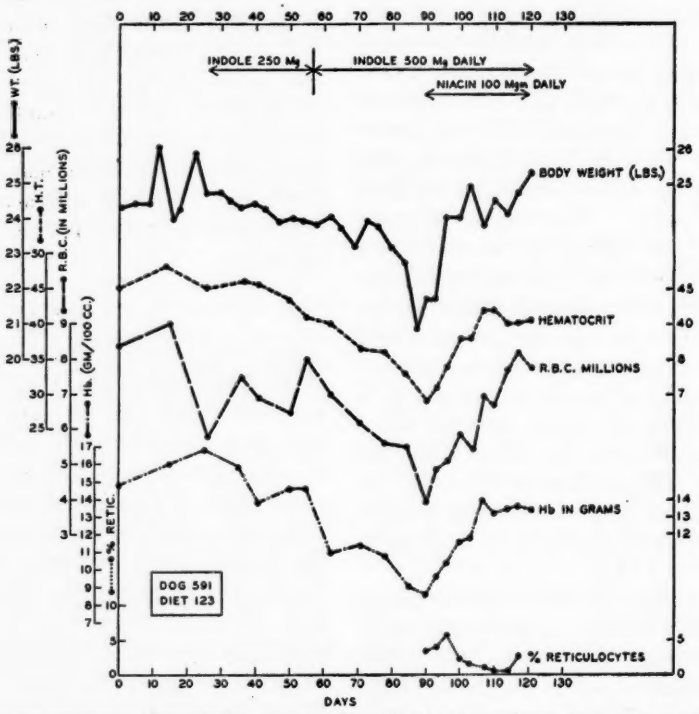

FIG. 1. EFFECT OF NIACIN on blood changes produced by indole given to a dog on Goldberger Diet No. 123.

Although the primary factor required for response appears to be niacin, there are indications that the condition may be much more complex. Thus, there is no characteristic reticulocyte response to niacin. But 2 dogs made anemic with this technique were treated with a purified liver extract (Reticulogen) furnishing 20 units of antipernicious anemia factor and less than 5 mg. of niacin daily. One dog showed a definite blood response with 16 per cent reticulocytes in 4 days, but relapsed. The addition of 100 mg. of niacin daily gave a second response with 24.9 per cent reticulocytes, followed again by a relapse. Daily 5 mg. of folic acid was then added with a third reticulocyte response to 32.8 per cent, but no rise in hematocrit or hemoglobin. The animal died shortly thereafter, apparently due to the large dose of indole (1 gm. daily) (fig. 2).

The second dog showed no response after 7 days of purified liver extract and had only a questionable rise in reticulocytes to 4.1 per cent. With niacin in addition to the 'Reticulogen' there was a slow response, with a reticulocyte peak of 6.9 per cent and an eventual hematocrit of 39 after 21 days.

A third dog treated with 100 mg. of niacinamide showed no significant response after 14 days, but reached a hematocrit of 41 in 71 days after being given 20 units of 'Reticulogen' daily while the niacin was continued.

Thus a factor present in purified liver extract containing practically no niacin appears to be involved in some of these dogs.

The possible role of tryptophane is again suggested by the fact that tryptophane is the only naturally occurring amino acid which is an indole derivative. Since there is much experimental evidence that tryptophane can be converted into niacin, 2 of the anemic dogs were treated with

tryptophane in doses of 1 gm. and 2 gm. of DL-tryptophane daily. Neither responded favorably.

Thus it appears that the indole effect is not an interference with normal tryptophane metabolism or conversion of tryptophane to niacin. The direct antagonism of indole by niacin. The many questions involved offer interesting possibilities.

You have no doubt noted how tryptophane has appeared in this discussion, first in connection with the anemia of pyridoxine deficiency,

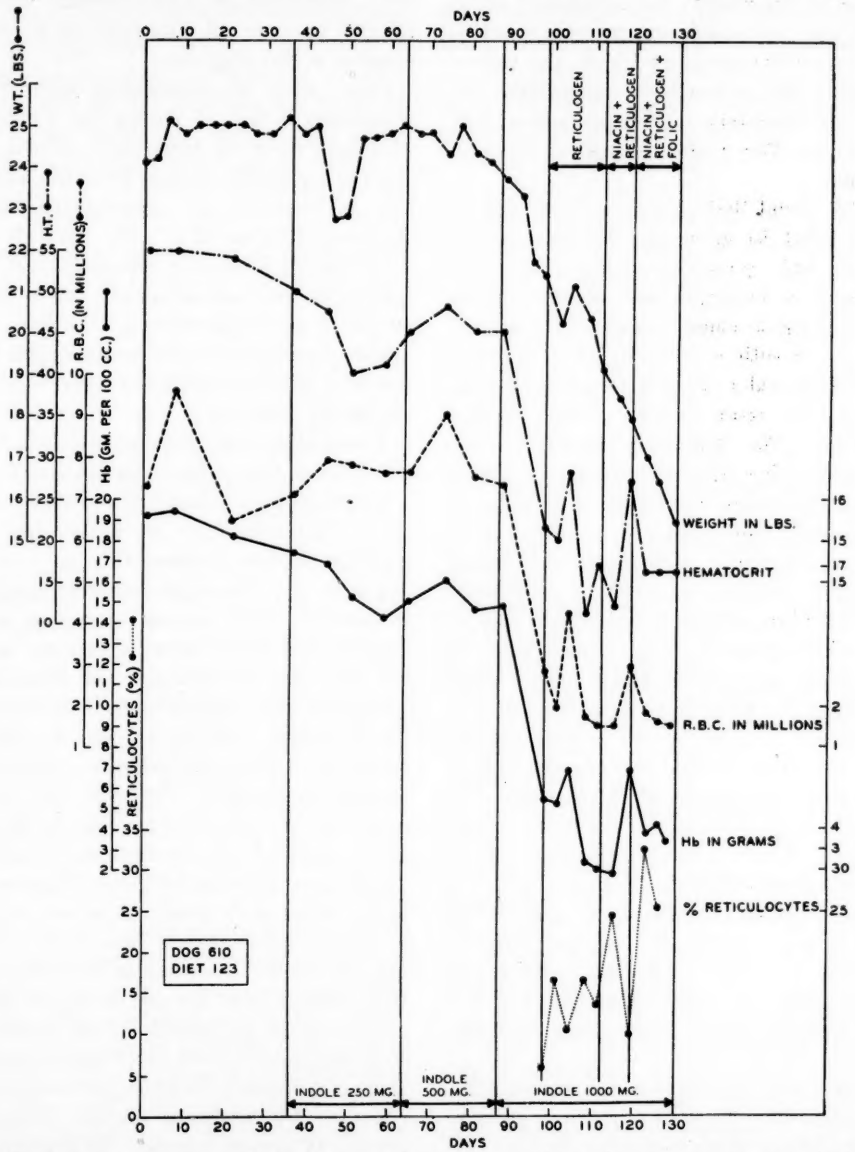

FIG. 2. EFFECT OF 'RETICULOGEN,' niacin and folic acid on blood changes produced by indole given to a dog on Goldberger Diet No. 123.

mechanism of the response to niacin is not clear. Niacin may be an essential element in blood formation, and when indole given with a niacin deficient diet results in hemolytic anemia, niacin may become a limiting factor in erythrocyte formation. There is also the possibility of a

and again here in connection with niacin deficiency. This will be mentioned further in the discussion of amino acids.

AMINO ACIDS

The production of anemia on diets low in protein is too well known to warrant review here.

This really is to be expected if it is remembered that globin, a sulfur containing protein, constitutes more than 90 per cent of hemoglobin (27). The physiological importance of this is indicated by the production of blood from body protein in the anemic dog during protein deprivation (45). In spite of its importance, the complexity of the problems involved still leaves us very much in the dark as to the specific roles of the various amino acids, the presence of unidentified factors, or the metabolic interrelationship with other factors. The evidence suggests all these mechanisms.

Daft (46) found that rats on a 4 per cent casein diet failed to grow and became anemic, leukopenic and granulocytopenic. Such animals showed a leucocyte and granulocyte response following treatment either with folic acid, vitamin B_{12}, or with at least one other factor in liver. He obtained a complete response following treatment with extra protein or the essential amino acids. The deficiency condition could be prevented either by increasing the protein in the diet or by giving a mixture of methionine, threonine and tryptophane.

These observations suggest several impor- that possible interactions, and the mechanism by which the low protein diet results in a blood dyscrasia accompanied by a deficiency or depletion of folic acid and B_{12} is not clear. Daft's data support the view that these vitamins may be synthesized from the amino acids in the animal and that this relation between amino acids and vitamins may account for the findings. His data show that although methionine, tryptophane, and threonine have preventive action, they alone are of little value in treatment. The simultaneous administration of folic acid, PABA or of isoleucine, valine, and histidine corrected the dycrasia in a large proportion of the experimental animals. It appears that folic acid may be synthesized in animal tissue under these conditions.

Wintrobe *et al.* (27) have shown in swine that a diet apparently deficient only in protein resulted in anemia which responded to folic acid. Shehata and Johnson (47) have fed rats a low protein diet containing sulfathalidine and have shown that the incidence of anemia under these conditions depends on the level of protein in the diet.

Daft (46) has suggested the possibility that amino acid imbalance or protein deficiency may be concerned in some human blood dyscrasias. There are old clinical observations suggesting that tyrosine metabolism is concerned in human pernicious anemia (48–50). Recent work by Swendseid *et al.* (51) based on the excretion of phenols in this disease supports this viewpoint. The fact that folic acid, pyridoxine, and ascorbic acid deficiencies all affect tyrosine metabolism (52–54) and are all concerned in experimental anemias is also suggestive.

Other work of considerable interest in this connection is that of Fontes and Thivolle (55), who have observed therapeutic benefit in some cases of pernicious anemia from the administration of histidine and tryptophane. Although Robscheit-Robbins *et al.* (56) have shown that no one amino acid is the essential one in erythropoiesis, it does not necessarily follow that they are all of equal importance. We have recently obtained some interesting results in this field and the data which I shall now present has not been previously reported.

Young albino rats were fed a protein free basal ration for 5 days while being subjected to severe hemorrhage by a technique previously described (57). On the fifth day the protein free ration is replaced by a test ration differing only in that mixtures of amino acids replace an equal weight of dextrose. The mixture of amino acids used was based on the 10 essential ones for rat growth (58, 59). One diet contained all 10 amino acids. The other diets differed only in the omission of the indicated amino acid. Results in table 1 are given on 10 rats in each group which survived 10 days on the diet. Although only the results on hematocrit values are given in the table, hemoglobin and red cell determinations gave values which agree with the hematocrit figures. These observations with those on serum protein will be reported elsewhere in more detail. The findings are most interesting in that they indicate that absence from the diet of any of the amino acids essential for growth except arginine causes some interference with blood regeneration under these conditions. However, the variation in the effect of the omission of the various amino acids is of greater interest. It is apparent that the omission of valine or histidine most seriously handicaps blood regeneration. On the valine free diet there was great difficulty in securing 10-day survivors, and we were able to include only 9 animals in this particular test. The results with the histidine deficient diet indicate the great importance of this amino acid in hemoglobin and erythrocyte production. This is to be

expected in view of the known high histidine content of hemoglobin (60, 61). It was rather surprising that dietary methionine and tryptophane deficiency resulted in such slight interference with blood regeneration. However, the important points I wish to make are that there are apparent variations in the importance of amino acids in blood formation; that valine and histidine are especially concerned; and that a dietary deficiency of any one of the 10 essential amino acids for growth, with the exception of arginine, causes some interference with blood regeneration following hemorrhage in rats on a protein free diet.

TABLE 1. AVERAGE CHANGES IN HEMATOCRIT ON AMINO ACID DIETS FOLLOWING HEMORRHAGE

DIET	AMINO ACID OMITTED	HEMATOCRIT			t
		Days		Increase in 10 days	
		0 Vol. %	10 Vol. %	= S. D. Vol. %	
1305		14.2	43.8	29.6 = 2.4	
1337	Valine	15.0	29.1	14.1 = 1.3	5.0
1356	Leucine	14.1	34.6	20.5 = 2.5	2.2
1407	Tryptophane	14.9	37.7	22.8 = 2.0	1.8
1408	Histidine	14.5	22.8	8.3 = 2.7	5.2
1409	Isoleucine	14.0	39.4	25.4 = 2.0	2.2
1410	Threonine	14.4	35.9	21.5 = 1.7	2.4
1411	Methionine	14.2	39.1	24.9 = 1.8	1.3
1412	Phenylalanine	14.4	34.4	20.0 = 1.7	2.8
1413	Arginine	14.6	42.7	28.1 = 1.0	.3
1414	Lysine	14.6	32.6	18.0 = 2.2	3.2

't' values of 4.0 indicate significant results 999 times in 1000 trials.

VITAMIN C

The only other vitamin except those of the B complex which appears to be intimately concerned with erythropoiesis is vitamin C. Although anemia has been found in experimental animals with scurvy (62), opinion has been divided as to whether a true anemia occurs with human vitamin C deficiency (63, 64). The weight of evidence is much in favor of a role for vitamin C in erythropoiesis, which, however, may be a complex one as is the case with the other vitamins. This is indicated by the work of Dyke *et al.* (65) who observed cases of pernicious anemia which failed to respond satisfactorily to adequate doses of potent liver extract unless vitamin C was administered. These cases probably were receiving an insufficient amount of vitamin C in their diets.

Vilter has recently reported (66) observations on 26 cases of uncomplicated scurvy of which only 2 had no anemia. He concludes that vitamin C is essential for normal hematopoiesis. Some of the characteristics of the anemia suggest to him that hemolysis may be a factor and that there is also a depression of bone marrow activity.

The question of the relation of vitamin C to erythropoiesis is further complicated by the observation that a macrocytic anemia sometimes occurs in patients with vitamin C deficiency and responds to the administration of vitamin C (67). The entire problem of the mechanism of action of vitamin C in erythropoiesis seriously needs further study. This is especially suggested by the report of Woodruff and Darby (54) that tyrosine metabolism in the scorbutic guinea pig can be favorably influenced by either vitamin C or pteroylglutamic acid.

MACROCYTIC ANEMIAS

Many new opportunities for research on the macrocytic anemias have occurred as a result of the discovery of the pteroylglutamates and vitamin B_{12}. There is such intense activity and rapid progress in this field that much of what I say today may be made out of date by work reported during this meeting.

The literature on the pteroylglutamates is already enormous and would require a monograph in itself. I shall not even attempt a superficial review of such an extensive and important mass of material, but rather shall select just a few points of special current interest. The truly brilliant research involved in the discovery, isolation and synthesis of folic acid, together with the steadily growing body of knowledge concerning its physiology, has been covered in previous reviews (68–70).

It is now clear that the pteroylglutamates are essential for a variety of animals and are found widely in plants. In man they are effective therapeutic agents in sprue, in nutritional macrocytic anemia, in the macrocytic anemia of pregnancy, in the megaloblastic anemia of infancy, and in securing a hematopoietic response in pernicious anemia (71–73).

The clinical activity of the pteroylglutamates overlaps that of vitamin B_{12}, although the 2 substances are quite obviously entirely different chemically. The most important clinical differ-

ence is that the pteroylglutamates have no beneficial effect on the central nervous system lesions of pernicious anemia (74–76), although these lesions are adequately controlled by vitamin B_{12} (77).

In spite of the demonstrated therapeutic effect of the pteroylglutamates on human macrocytic anemias, there is as yet no proof that added folic acid is needed by normal persons. Although estimates of the quantity needed have been made on the basis of excretion studies (70, 78), such estimates do not tell us much about the normal human requirement or the controlling conditions. The solution of such problems must await better knowledge of the mechanism of action and relation to other substances essential in erythropoiesis.

I have already mentioned Daft's evidence suggesting that folic acid may be synthesized by the rat. If it can be synthesized by other mammals, this observation may be of great significance. There is great research interest in the relation of folic acid to other nutrients as well as to its mechanism of action, and it has been suggested that it is concerned in the metabolism of purines and pyrimidines (79, 80).

Elvehjem and his associates have studied the relation between folic acid and niacin deficiency and anemia in the dog (81). There are many implications of importance in their observations. The recent paper of Ruegamer *et al.* (82) reports on young dogs fed a niacin deficient diet plus sulfasuxidine. Niacin therapy was only partially successful. Folic acid brought about a better response to niacin, but had no effect on a macrocytic anemia which developed progressively. One USP unit of 'Reticulogen' brought about complete recovery. Other liver extracts were only partially effective unless given in combination with folic acid. There was no need for folic acid if high levels of protein were used. One of the important points in this work was that several of the dogs developed a flaccid paralysis, suggesting that these workers may be opening the way to study of the neurological changes found with some macrocytic anemias for which we now have no explanation.

One of the most interesting developments in connection with the pteroylglutamates is the synthesis and action of inhibitors or antagonists. Jukes (83) has described seven pteroylglutamic acid antagonists synthesized by different workers. Some of these compounds have proven especially useful in acute leukemia of children, although they are toxic and must be handled with care (84).

These compounds offer important research possibilities, and, according to the metabolite-antimetabolite theory of inhibitors, offer a chance to find further substances. This also suggests the possibility of inhibitors for vitamin B_{12}, which no doubt would assist in clarifying the action of that substance.

In this connection, I would like especially to call attention to the possibilities offered by study of the macrocytic anemia due to infestation by the fish tape worm. This anemia has been reported to respond to treatment with anti-pernicious anemia liver extract. This, therefore, offers another line of approach to the etiological factors involved in the macrocytic anemias, and I am surprised that this unusual anemia has not attracted more special study of a fundamental nature. It seems quite possible that the fish tape worm may elaborate an inhibitor or antagonist, although von Bonsdorff (85) failed to obtain any evidence of such action with dried tape worm.

Cartwright *et al.* (86, 87) have produced a macrocytic anemia in swine using a pteroylglutamic acid antagonist (methyl folic acid) in the presence of one USP unit of purified liver extract. There was rapid response to pteroylglutamic acid, but response to vitamin B_{12} or purified liver extract was less than with pteroylglutamic acid. Thymine and xanthopterin had little activity and tyrosine, adenine, and uracil were inactive.

Finally, we came to one of the greatest advances in the field of erythropoiesis. This was the isolation from liver of vitamin B_{12} in 1948 by Rickes *et al.* (88) and by Smith (89). This discovery has already produced a voluminous literature and stimulated research on anemia to a degree never before seen.

Vitamin B_{12} is reported to be a red crystalline compound with a molecular weight probably between 1500–1600, containing cobalt, phosphorus, and nitrogen (90, 91). This material is now the most potent known therapeutic substance. West (92) has obtained a maximal reticulocyte response on pernicious anemia with one microgram daily, and 25 mcg. once a week has resulted in normal blood counts and improvement in spinal cord lesions. Cobaltous ion in doses of 150 mg. was ineffective, and 5.4 mg. of thymidine was practically without effect (93).

This observation is of particular interest be-

cause of the results of bacterial studies. Stokes (79) has found that with certain bacteria thymine (5-methyl uracil) may substitute for pteroylglutamic acid. Prusoff, Tepley and King (94), using a medium partially deficient in pteroylglutamic acid but otherwise satisfactory for Lactobacillus casei, obtained a lower content of desoxyribonucleic acid without any change in the content of ribonucleic acid. An excess of thymine caused a moderate rise in both desoxyribonucleic acid and ribonucleic acid.

Shive *et al.* (95) have shown that thymidine (thymine desoxyriboside) may substitute for liver extracts with antipernicious anemia activity and presumably the substitution was for vitamin B_{12}.

Wright (96) has found that with some lactic acid bacteria desoxyribonucleic acid, but not ribonucleic acid, may substitute for vitamin B_{12}; and has postulated that vitamin B_{12} functions in the conversion of thymine to thymidine.

Frommeyer and Spies (97) have shown a hematopoetic effect in human macrocytic anemia from large doses of thymine, and Spies *et al.* (98) report successfully treating macrocytic anemias in relapse first with thymine, on second relapse with folic acid, and on third relapse with vitamin B_{12}, thus indicating the overlapping of these widely different substances therapeutically, but in very greatly differing doses. On the basis of the massive doses of thymine used by Spies, it is quite possible that the failure of thymidine in West's hands may have been due to the relatively small dose. It will be interesting to see the results when larger amounts can be tested.

There is considerable evidence that vitamin B_{12} is identical with at least one animal protein factor (99–101). Further work will be required to determine whether the essential chick growth factor concentrated from fish products by Pensack, Bethke and Kennard (102) is identical with vitamin B_{12}.

Although vitamin B_{12} is effective in controlling pernicious anemia, nutritional macrocytic anemia and sprue (98), Bethell *et al.* (103) have reported a case of macrocytic anemia of pregnancy which did not respond to vitamin B_{12} but did respond to intramuscular pteroylglutamic acid. It is not yet clear whether this is an unusual case similar to the few cases of sprue and pernicious anemia which fail to respond to pteroylglutamic acid, or whether vitamin B_{12} will prove to be ineffective in this type of macrocytic anemia. Luhby (104), studying the megaloblastic anemia of infancy which responds to pteroylglutamic acid, failed to secure a response with vitamin B_{12} alone and suggests that both vitamin B_{12} and folic acid are normally involved in the protein metabolism of blood formation.

Bethell *et al.* (105) have made the interesting observation that the feces of normal persons and of pernicious anemia patients in relapse contains a substance which promotes the growth of test organisms for vitamin B_{12} in approximately the same amount. Furthermore, he has produced a significant response in patients with pernicious anemia by injecting an extract of feces from patients with untreated pernicious anemia. These findings suggest that one of the factors in pernicious anemia may be an inability to absorb vitamin B_{12}, or that vitamin B_{12} is produced by bacterial action in the intestine and is unavailable for absorption.

Castle (106) reports that gastric juice potentiates the effect of oral vitamin B_{12}, but the activity is not as great as when vitamin B_{12} is given intramuscularly without gastric juice. Vitamin B_{12} and extrinsic factor may be identical, and the action of the intrinsic factor may be to promote absorption of vitamin B_{12} rather than to act upon the extrinsic factor in food.

Thus the availability of vitamin B_{12} has, by moving us ahead, presented a multitude of opportunities to solve new problems. It is leading rapidly to further studies of the metabolism of nucleic acids.

It is a tribute to the alertness of the planners of the Federation Meetings that a symposium on the chemistry and metabolism of nucleic acids and their constituents was held at the 1947 meeting (107) when the possible importance of this subject in the anemias could not be clearly foreseen.

The activity of ventriculin still must be explained, and finally, there is the possibility of additional new factors—such as the vitamin B_{14} which Norris and Majnarich (108) have obtained from human urine and which appears to be exceedingly potent in accelerating bone marrow proliferation. This substance does not contain cobalt. Hays (109) has obtained evidence of another factor in liver affecting in vitro red blood cell maturation which he believes is not vitamin B_{12}.

It is clear that the field is broad, progress rapid and that much remains to be explained.

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American Association of Immunologists

IVORY PAWN IN THE IVORY TOWER[1]

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NEW YORK CITY

UNTIL recently it was the privilege of the scientist to hold himself aloof from the mundane cares and problems of the business man, the politician, the diplomat and the soldier, and to pursue his butterfly, his gene, his diplococcus, or his macromolecule relatively undisturbed by the rumblings and creakings of the outside world. But two global wars in two generations have made this isolation dangerous, if not impossible. Many of his kind have been engaged in applying his very science to more and more efficient, less and less selective methods of mass destruction. Others have brought the far ends of the earth closer by air than are our most distant cities by train, so that all the peoples of the globe are neighbors. The economic dislocations of two wars and the unrest born of them have reached into the hospital, the university, and into the laboratory of our hitherto aloof scientist. Where will the money come from for the life-blood of his work, where can he publish his data, and will they actually be speeded to the far corners of the earth, now only a few air-hours away? How much individualism, if any, shall we give up in our domestic planning?—shall there be a National Science Foundation?—how much sovereignty or international individualism shall we give up in order to avoid the unspeakable catastrophe of another war and attempt to ensure an era of fertile peace?

These problems crowd upon our scientist, for he must protect and preserve those he loves even as he must strive for the continuity of his work, so seriously threatened. I shall leave to others consideration of our internal political and economic problems and confine myself entirely to international relations, which determine whether we are to be at peace with our neighbors and can work on in peace, or whether we are to be sucked into another war which will surely make an end to our aspirations. What do you and I know about the foreign policy of our nation or of other nations, what can you and I do to know more about these international relations, and what can you and I do to exert our influence for fair play, for justice and for peace?

You and I, as scientists, interest ourselves in our foreign policy not only as a matter of self-preservation, but also because of our duty to do so by virtue of our special training. We are accustomed to reason clearly, to balance cause and effect, to bring every resource to bear upon the elusive detail which clears up the whole mystery. Equally important, through years of practice and discipline, we have formed the habit of self-criticism, and knowing our own weaknesses and limitations, we can the more readily see those of others. Added to these attributes of maturity is an enduring patience, the knowledge of when and how to watch and listen. What better equipment could there be for a critical examination of our foreign relations?

Last September, in Paris, I spent 10 days listening to the General Assembly of the United Nations and its Political and its Social and Economic Committees, where were gathered the diplomats, the politicians, the experts and the soldiers of the nations of the world. Only rarely did I hear evidence of those qualities of the human mind which we, as scientists, admire and honor in the practice. Were we to abandon these, science could not progress and, I submit, the construction of a durable peace will be equally impossible if emotions are appealed to rather than reason, and if partisanship, the emphasis on prestige, and the unwillingness to admit fallibility are not abandoned. It is in this area that our special qualifications can make our influence felt, and

[1] Presidential Address, Detroit, April 19, 1949.

there are several organizations and activities which can help us be directly useful.

How many of you are members of the American Association for the United Nations? How many are members of the Foreign Policy Association? How many belong to foreign policy study groups of the League of Women Voters? These organizations publish bulletins and hold meetings which will keep us informed and provide opportunities and occasions for our active participation in the search for the quiet atmosphere of peace without which our scientific effort will be wasted and doomed. But, you say, what can you and I do, except to read books and pamphlets and answer questionaires, even within these groups?

While the United Nations Organization is one of governments, the American Association for the United Nations represents the people of the United States in their aspirations for the United Nations. The American Association for the United Nations is a member of the World Federation of United Nations Associations, which meets every year, and where delegates from some forty national associations for the UN discuss common problems of building an alert public opinion on the UN and plan the measures they would like to see the UN take. The Federation has consultative status A, as it is called, which means that the Economic and Social Council of the United Nations and its Commissions must receive and consider its recommendations. I have been a delegate from our Association these last two years, and in this way, some of you, too, could make the influence of your scientific judgment felt. This year there were delegations from such central European countries as Czechoslovakia, Poland, Hungary, Bulgaria, and Roumania present among the others, and although some of their members were belligerent and antagonistic at the start, all of our resolutions and recommendations were, in the end, unanimously adopted in the various working commissions and at the final plenary session. Clearly, *people* of divergent views can often agree even when their *governments* fail to find a common basis for action. Two of our 1947 resolutions were unanimously adopted, with only slight modification, by the General Assembly of the United Nations when it convened in New York in the winter of 1947–8, and until one of these was considered, the General Assembly had been unable to agree upon anything. As stated at the time by Dr. Evatt, of Australia, this changed the whole atmosphere of the meeting for the better. The General Assembly, as you see, is quick to respond to constructive popular suggestions—their implementation, which so far has rested with the individual governments, is another matter.

Now that the more moderate portion of the World Federalists has decided to work for the evolution of the United Nations into a world government there should be large areas in which cooperation of the World Federalists and the American Association for the United Nations should be feasible, including participation in the work of the World Federation of United Nations Associations.

Another international activity in which you and I can participate is the United Nations Appeal for Children, which will continue under the United Nations International Children's Emergency Fund, or UNICEF. This work was organized by Mr. Aake Ording, of Norway, so that everyone could take part by giving food or a day's work or its equivalent, and so strive actively in a world-wide cooperative effort for the relief of suffering. We can also give aid of books, materials or funds to the Pestalozzi Village in Trogen, Switzerland, where orphans from the devastated countires, often demoralized and brutalized by their experiences, are given a quiet family life in the cultural atmospheres of their own nations while attempts are made to solve their psychological problems. The French have a similar Ecole Médico Pèdagogique at Nivillers, Oise, for orphans of the Résistance, and this effort is desperately in need of aid.

As you see, it is not only by our membership in international scientific societies that we can promote exchanges among peoples and can help to lessen the international tensions and rivalries that have hitherto led to wars, but must be allowed to do so no longer. You and I, as scientists, have a special fitness and a special aptitude, born of long practice and tradition, for this work, and we cannot hold aloof from it without inviting forfeiture of the rights and privileges of science and our privileges as scientists. These rights and privileges are in grave danger. As immunologists we have always preferred active intervention to passive immunization. I have suggested several available vaccines—you may grasp at these or reach for others before the onset of chills and fever. Bring on the hypodermics! The time is short in which to bestir ourselves as active, thoughtful, informed, critical, yes, patriotic citizens.